ANTI-INFLAMMATORY EFFECTS OF INHIBITORS OF THE NF-κB PATHWAY IN THE MOUSE ASTHMA MODEL

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SUMMARY

The NF-κB family is a central player in coordinating both innate and adaptive immunity and is involved in the regulation of a broad array of genes in response to diverse stimuli. The NF-κB family also plays a key role in the initiation and development of asthma. Because the NF-κB transcription factors are central to both normal biological functions and pathological conditions, absolute inhibition of NF-κB per se may not be a safe approach. Rather, appropriate and specific inhibition of signaling molecules that regulate NF-κB activity may be an effective anti-inflammatory strategy for asthma. The objectives of my thesis project were to examine the potential anti-inflammatory effects of a GSK-3β inhibitor, namely TDZD-8, and a herbal medicinal, namely andrographolide in a mouse asthma model and elucidate their mechanisms in the regulation of NF-κB pathway.

BALB/c mice sensitized and challenged with ovalbumin developed allergic airway inflammation. Intravenous administration of TDZD-8 significantly ($P < 0.05$) inhibited ovalbumin-induced increases in total cell counts, eosinophil counts, IL-5, IL-13, and eotaxin levels in bronchoalveolar lavage fluid, and OVA-IgE in serum. In addition, TDZD-8 reduced ovalbmuin-induced increase in mRNA levels of inflammatory molecules, infiltration of inflammatory cells, and mucus hypersecretion in lungs. TDZD-8 also suppressed airway hyperresponsiveness to methacholine in mice. Western blotting of the whole lung and human bronchial

epithelial cell showed that TDZD-8 may exert its anti-inflammatory effects by inhibiting the phosphorylation of p65.

Andrographolide attenuated inflammatory cell counts, IL-4, IL-5, IL-13, and eotaxin levels in bronchoalveolar lavage fluid, concentration of total IgE, OVA-IgE, OVA-IgG1 in the serum, and expression of inflammatory molecules in the lung, in a mouse asthma model. Andrographolide also suppressed OVA-induced infiltration of inflammatory cells and mucus hypersecretion in the lungs, and OVA-induced airway hyperresponsiveness to methacholine. Western blotting and TransAM assay suggested that andrographolide may exert its anti-inflammatory effects by inhibiting the phosphorylation of IKKβ and suppressing the DNA-binding activity of p65.

Taken together, these present findings implicate that appropriate and specific inhibition of signaling molecules that regulate NF-κB pathway may have therapeutic potential for the treatment of allergic airway inflammation.

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LIST OF ABBREVIATIONS

AHR	airway hyperresponsiveness
ALT	alanine aminotransferase
AMCase	acidic mammalian chitinase
AMV	avian myeloblastosis virus
AP	alkaline phospatase
AP-1	activator protein-1
APC	antigen presenting cell
ASM	airway smooth muscle
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
bFGF	basic fibroblast growth
BSA	bovine serum albumin
cAMP	adenosine 3′,5′-cyclic monophosphate
CBP	CREB-binding protein
CCR	C-C chemokine receptor
Cdyn	dynamic compliance
Con-A	concanavalin A
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CS	inhaled corticosteroid
DC	dendritic cells
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
ELISA	enzyme-linked immunosorbent assay
ECP	eosinophilic cationic protein
ECL	enhanced chemiluminescent
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FcεRI	high affinity IgE receptor
FcεRII	low affinity IgE receptor
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/macrophage colony-stimulating factor
GR	glucocorticoid receptors
GRE	glucocorticoid-response elements
GSK-3	glycogen synthase kinase-3

HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecule-1
ICOS	inducible costimulatory protein
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	inhibitor of NF-κB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	janus kinase
JNK	c-Jun NH2-terminal kinase
LPS	lipopolysaccharide
LT	leukotriene
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MCP-1	monocyte chemoattractant protein
MEK	MAP/ extracellular signal-regulated kinase kinase
MHC	major histocompatibility complex
MMP	matrix metallopeptidase
NBT	nitroblue tetrazolium
NEMO	nuclear factor-κB essential modulator
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
Nrf2	nuclear factor-E2-related factor-2
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAF	platelet-activating factor
PAMP	pathogen-associated molecular pattern
PAS	periodic acid-schiff
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDE4	phosphodiesterase type 4
PE	phycoerythrin
PMBC	peripheral blood mononuclear cells
PVDF	polyvinylidene difluoride
RANTES	regulated upon activation, normal t-cell expressed, and secreted
REL	v-rel reticuloendotheliosis viral oncogene homolog
RHD	rel homology domain
RI	lung resistance

RT	reverse transcription
STAT	signal transducers and activators of transcription
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TCR	t-cell receptor
TDZD-8	4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
Th2	t helper2
TLR	toll-like receptor
TMB	3,3´,5,5´-tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TPL2	tumor progression locus 2
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
TTBS	tween-20 tris buffered saline
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
WNT	wingless and int-1

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

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Bao, Z., Lim, S. M., Liao, W. P., Lin, Y. Z., Thiemermann, C., Leung, B. P., and Wong, W. S. (2007). Glycogen synthase kinase-3beta inhibition attenuates asthma in mice. Am J Respir Crit Care Med 176, 431-438.

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1. INTRODUCTION

1.1. Asthma

1.1.1. Epidemiology of asthma

Asthma is a common chronic disease which affects around 300 million people of all ages and ethnic backgrounds. The prevalence of asthma is high in industrialized countries such as United Kingdom (15.3%), New Zealand (15.1%), Australia (14.7%), and United States (10.9%) when compared with non-industrialized countries, for instance, Mexico (3.3%), India (3%), and Iran (5.5%) (Masoli et al., 2004). A dramatic increase in the prevalence of asthma was reported in many countries from the 1960s to the 1990s (Eder et al., 2006). One popular theory which explains the rising prevalence of asthma today, especially in industrialized societies, is the "hygiene hypothesis". This hypothesis contributes the rising prevalence of asthma to the decreasing infection rates in children due to cleaner environments in industrialized countries. It is derived from the observation that the risk of hay fever varies inversely with family size, and is further supported by the phenomenon that exposure to microbial products released by farm animals exerts a protective role against the development of asthma (Strachan, 1989). Although asthma is generally not a life-threatening disease, mortality rates are still considerable, accounting for about 1 in every 250 deaths worldwide (Masoli et al., 2004). Furthermore, asthma is the third leading cause of hospitalization, exceeded only by pneumonia and injuries, among persons under 18 years of age in the United States (Eder et al., 2006). High hospitalization fee,

together with high incidence, lead to asthma related costs exceeding those of tuberculosis and acquired immunodeficiency syndrome (AIDS) combined, accounting for 1% to 2% of the total health-care budget in industrialized countries (Braman, 2006). Furthermore, the economic burden of asthma disproportionately affects uncontrollable asthma patients. In both western and developing countries, 10% to 20% uncontrollable asthma patients are responsible for approximately 50% of direct or indirect costs, whereas 70% of mild asthma patients account for only 20% of total costs (Beasley, 2002; Braman, 2006). In summary, the rising prevalence, mortality, and high economic burden of asthma are having huge effects on the health-care systems worldwide. Therefore, more research should be done to better understand the pathophysiology of asthma and further explore potentially effective therapies for this disease.

1.1.2. Susceptibility genes of asthma

Both genetic background (atopy) and environmental factors (allergens, viruses, and occupational exposures) contribute to the initiation and development of asthma (Busse and Lemanske, 2001). In developed countries, 30% of the population is atopic, whereas only 10-12% of the population suffers from asthma, suggesting that allergic responses to inhaled allergens are considered as risky factors rather than causative factors of asthma (Hammad and Lambrecht, 2008). Therefore, it is critical to identify both genetic and environmental factors and their interactions that might contribute to the development of asthma in a sensitized

subject. It has been more than 10 years since the first genome-wide screen for asthma and atopy susceptibility loci (Ober and Hoffjan, 2006). In a decade, rapid advances in identifying susceptibility genes for asthma have uncovered numerous genes which are crucial to the pathogenesis of asthma (Table 1.1) (Vercelli, 2008). These asthma susceptibility genes are involved in probably all aspects of asthma including innate immunity and immunoregulation, T helper 2 (Th2) cell differentiation and effector function, epithelial biology and mucosal immunity, lung function, airway remodeling, and disease severity (Vercelli, 2008). Despite the apparent achievements in asthma genetics, there remain huge confusing discrepancies about the linkage between genotypes and phenotypes of asthma. Both gene-environment and gene-gene interactions might dramatically change the impact of a specific gene on the complex phenotypes, as are supported by epidemiological studies of asthma (Moffatt et al., 2007; Vercelli, 2008). Finally, understanding of asthma genetics not only helps us unravel the pathogenesis of this disease, but may also provide information for pharmacogenetic approaches, leading to individualization of treatments with high efficacy and low side effects for patients (Hall, 2006).

1.1.3. Pathophysiology of asthma

Asthma is a chronic airway disease which is characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) (Figure 1.1) (Busse and Rosenwasser, 2003).

Table 1.1 Susceptibility genes identified for asthma (Adaped from Vercelli, 2008)

Gene	Chromosome	Function and pathway
GSTM1	1p13.3	Environmental and oxidative stress-detoxification
FLG	1q21.3	Epithelial barrier integrity
IL10	1q31-q32	Immunoregulation
CTLA4	2q33	T-cell-response inhibition and immunoregulation
IL-13	5q31	Th2 effector function
IL-4	5q31.1	Th2 differentiation and IgE induction
CD14	5q31.1	Innate immunity-microbial recognition
SPINK5	5q32	Epithelial serine protease inhibitor
ADRB2	5q31-q32	Bronchial smooth-muscle relaxation
HAVCR1	5q33.2	T-cell-response regulation-HAV receptor
LTC4S	5q35	Cysteinyl leukotriene biosynthesis, inflammation
LTA	6q21.3	Inflammation
TNF-α	6q21.3	Inflammation
HLA-DRB1	6q21	Antigen presentation
HLA-DQB1	6q21	Antigen presentation
HLA-DPB1	6q21	Antigen presentation
GPRA	7q14.3	Regulation of cell growth and neural mechanisms
NAT2	8p22	Detoxification of drugs and carcinogens
FCERIB	11q13	High-affinity Fc receptor for IgE
CC16	11q12.3-q13.1	Epithelium-derived anti-inflammatory protein
GSTP1	11q13	Environmental and oxidative stress, detoxification
IL-18	11q22.2-q22.3	Induction of IFNγ and TNF
STAT6	12q13	IL-4 and IL-13 signalling
NOS1	12q24.2-q24.31	Nitric oxide synthesis — cell–cell communication
CMA1	14q11.2	Mast-cell chymotryptic serine protease
IL-4R	16p12.1-p12.2	α-chain of the IL-4 and IL-13 receptors
CCL11	17q21.1-q21.2	Epithelium-derived eosinophil chemoattractant
CCL5	17q11.2-q12	Monocyte, T-cell and eosinophil chemoattractant
ACE	17q23.3	Inactivation of inflammatory mediators
TBXA2R	19p13.3	Smooth-muscle contraction, inflammation
TGFB1	19q13.1	Immunoregulation, cell proliferation
ADAM33	20p13	Cell–cell and cell–matrix interactions
GSTT1	22q11.23	Environmental and oxidative stress,detoxification

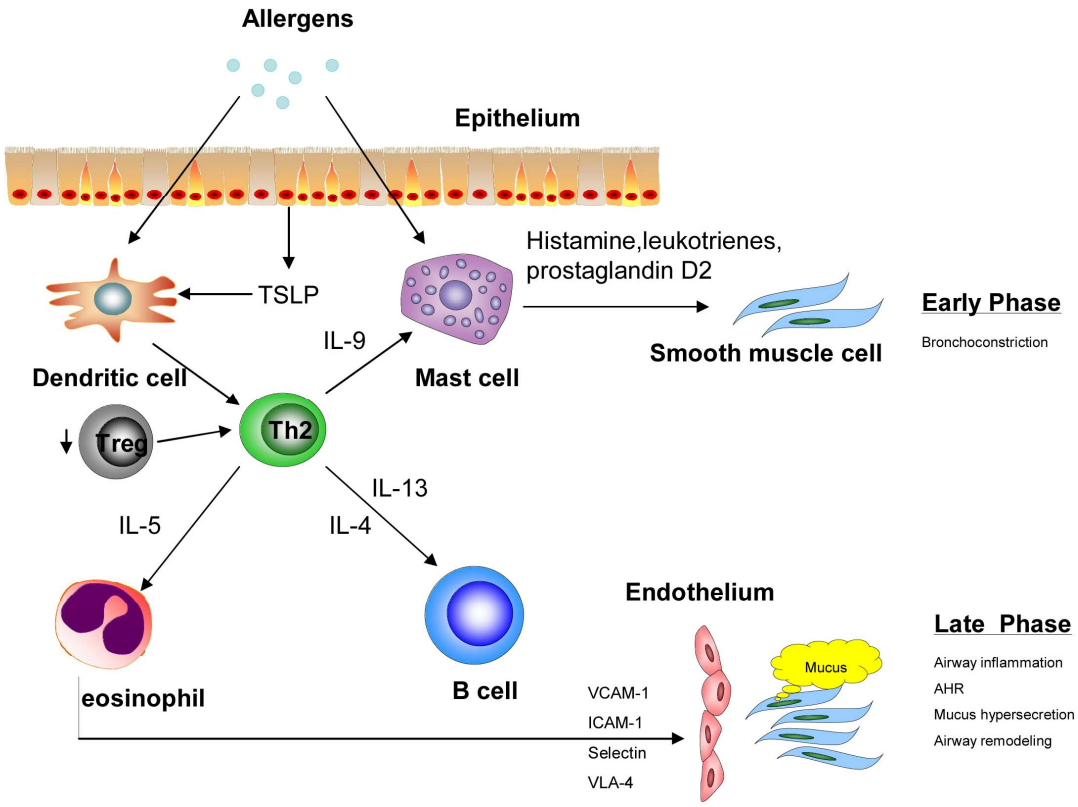


Figure 1.1 Schematic diagram of pathogenesis of asthma. Definition of abbreviations: ICAM-1 = intercellular adhesion molecule-1; TSLP = thymic stromal lymphopoietin; VCAM-1 = vascular cell adhesion molecule-1; VLA-4 = very late antigen-4.

Inhaled allergens, often the initiator of the asthma, are taken up by lung dendritic cells (DC). Then, under the presence of low concentration toll-like receptor (TLR) agonist within the allergen itself or the presence of proteolytic activity within the allergen, DCs are activated and migrate to the draining lymph nodes where they present allergens to naïve CD4+ T cells, promoting the differentiation of naïve CD4+ T cells into Th2 cells (Hammad and Lambrecht, 2008). Th2 cells have a central role in the pathogenesis of asthma and produce an array of cytokines such as interleukin-4 (IL)-4, IL-5, IL-9, and IL-13. IL-4 is mainly responsible for the B cells isotype switching. Under the presence of IL-4, IL-13, and other molecules, B cells undergo isotype switching and synthesize IgE which is released into circulation, eventually binding to high affinity IgE receptors (FcεRI) on the surface of mast cells. Crosslinking of antigens, IgE, and FcεRI on mast cells lead to the degranulation of mast cells and the release of mediators including histamine, leukotrienes, and cytokines, causing acute bronchoconstriction (Busse and Lemanske, 2001). IL-5 is the most critical cytokine mediating the differentiation, activation, and survival of eosinophils, which may contribute to both inflammation and airway remodeling in asthma (Simon and Simon, 2007). IL-9 could promote the proliferation of mast cells. Furthermore, IL-13 is the most pivotal effector of all Th2 cytokines, inducing almost all pathophysiological features of asthma comprising airway inflammation, AHR, mucus oversecretion, and airway remodeling (Wills-Karp, 2004). In addition, adhesion molecules, their

receptors, and chemokines are vital for the transmigration of inflammatory cells from circulation into inflammatory sites in response to allergic provocation (Rosenberg et al., 2007). Infiltration of inflammatory cells and the release of Th2 cytokines may lead to transient and reversible AHR, whereas multiple structural changes in the airway, known as airway remodeling, could contribute to persistent AHR (Cockcroft and Davis, 2006).

1.1.3.1. Mast cells

Mast cells arise from CD34+ pluripotent stem cells in the bone marrow, circulate in the blood as precursors, and then undergo tissue-specific maturation. In tissue, mast cells mature under the influence of stem cell factor (SCF) and its receptor CD117. In addition to SCF, mast cell growth and differentiation is manipulated by various cytokines, including IL-3, IL-4, IL-6, IL-9, IL-10, and nerve growth factor (Brown et al., 2008).

Mast cells are activated by the crosslinking of FcεRI or by non-IgE-mediated pathways through complement receptors or toll-like receptors. Upon activation, mast cells release an array of mediators, cytokines, and chemokines. The pattern of mediator release is modulated by cytokines, growth factors, and the microenvironment (Brown et al., 2008). For instance, IL-4 could augment FcεRI-mediated responses by mast cells (Bischoff et al., 1999), whereas, IL-10 and transforming growth factor-β (TGF-β), produced by regulatory T cells could diminish those reactions (Royer et al., 2001).

Mast cells release an array of mediators and cytokines which may induce inflammation, change airway smooth muscle (ASM) activity, lead to mucus hypersecretion, and cause Th2 polarization (Brown et al., 2008). Histamine, stored in the granules within mast cells, and lipid mediators such as leukotriene (LT)C4, LTD4, LTE4, and prostaglandin D2, all synthesized upon activation of mast cells, could induce contraction of ASM, causing bronchoconstriction. In addition, increased mast cells population, but not T cells or eosinophils population, was found in ASM of asthmatic patients (Brightling et al., 2002). Furthermore, activated mast cells produced growth factors (e.g. vascular endothelial growth factor [VEGF] and basic fibroblast growth factor [bFGF]), proteases, histamine, metalloproteinases, together with histamine and lipid mediators, could lead to proliferation and remodeling of epithelium and mucus hypersecretion. In addition, mucosal mast cells are recruited to the surface of epithelium by SCF, which has been shown to be overexpressed in the epithelium of asthmatic patients. Moreover, mast cell-derived cytokines such as IL-4, IL-5, IL-9, and IL-13 could lead to Th2 differentiation, causing allergic inflammation. Mast cells may contribute to both the early-phase and the late-phase reaction in asthma (Barnes, 2008; Brown et al., 2008).

1.1.3.2. Eosinophils

Eosinophil progenitors also arise from pluripotent CD34+ stem cells in bone marrow. Under the regulation of three classes of transcription factors including

GATA-1(a zinc finger family member), PU.1 (an Eta family member), c/EBP (CCAAT/enhancer-binding protein family), and cytokines such as IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF), eosinophils progenitors (CD34+IL-5R+) differentiate, mature and are released from the bone marrow into circulation where they represent 1% to 5% of the leukocytes. Under normal condition, the majority of eosinophils migrates into the gastrointestinal tract where they stay within the lamina propria of all segments except the esophagus (Hogan et al., 2008).

Eosinophils are involved in a variety of inflammatory processes, such as allergic diseases, parasitic infections, tissue injury and tumor immunity (Simon and Simon, 2007). In particular, elevated eosinophil count in tissue, blood, and bone marrow is one of the hallmarks of asthma and is associated with disease severity, suggesting that eosinophils are one of many pivotal effector cells in the pathophysiology of asthma (Bousquet et al., 1990; Hogan et al., 2008). In response to allergic stimuli, eosinophils are attracted to the inflammatory sites by the orchestration of Th2 cytokines (IL-5, IL-13), adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VACM-1] ,and selectin), chemokines(eotaxin-1,2,3, and regulated upon activation, normal T-cell expressed, and secreted [RANTES]), and other molecules (i.e. chitinases) (Rosenberg et al., 2007; Zhu et al., 2004). Of these cytokines, IL-5 is the most essential one which not only regulates the trafficking, but also the differentiation,

maturation, and survival of eosinophils. Eotaxins are eosinophil-specific chemokines which selectively regulate eosinophil trafficking through the C-C chemokine receptor (CCR)3 receptors, which are expressed predominantly on eosinophils. Briefly, eotaxin-1 is produced by epithelial cells upon stimulation by Th2 cytokines via signal transducer and activator of transcription (STAT)-6 dependent pathways. Both eotaxin-2 and eotaxin-3 are expressed at later time points following challenge by allergens as compared to eotaxin-1. In addition, polymorphisms in genes encoding eotaxin-2 and eotaxin-3 have been related to the increased eosinophil population in asthmatic patients (Rosenberg et al., 2007). Furthermore, IL-5 could synergize with eotaxins to enhance mobilization of eosinophils into the lung following allergen exposure.

At the inflammatory foci, eosinophils release numerous proinflammatory cytokines, lipid mediators (platelet-activating factor [PAF] and LTC4), and toxic granule proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin, and eosinophil peroxidase (Hogan et al., 2008). Eosinophils exert their functions, as one of major effector cells in allergic reactions, by secreting these inflammatory molecules which contribute to upregulation of adhesion systems, modulation of cellular trafficking, regulation of vascular permeability, mucus hypersecretion, smooth muscle constriction, even tissue damage and dysfunction (Hogan et al., 2008). Eosinophils can also modulate immune functions as an antigen presenting cell (APC) in addition to its

function as effector cells (Rothenberg and Hogan, 2006). In a mouse asthma model, eosinophils from allergic lungs expressed both classes of major histocompatibility complex (MHC) Ⅰ and Ⅱ peptides and T cell costimulatory molecules (CD80 and CD86), migrate to regional lymph nodes, and functioned as APCs to stimulate CD4+T cells. Blockade of CD80 and CD86 by monoclonal antibodies diminished eosinophil-dependent T cell proliferation and cytokine secretion (Shi, 2004). In addition, recent evidence has suggested that eosinophils may be involved in airway remodeling in asthma (Foley et al., 2007). Eosinophils are a significant source of profibrotic cytokines and fibrogenic mediators including TGF-β, IL-11, IL-17, TGF-α, and matrix metallopeptidase (MMP). In particular, TGF-β stimulates fibroblast to promote the synthesis and secretion of numerous proteins into the extracellular matrix. The thickening of the subepithelia basement membrane has also been shown to be related to the infiltration of eosinophils in bronchial mucus in severe asthma patients (Foley et al., 2007). Moreover, one study has shown that there are TLRs on human eosinophils, and peptidoglycan (TLR2 ligand), flagellin (TLR5 ligand), and imiquimod R837 (TLR7 ligand) trigger both nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in eosinophils, leading to the release of IL-1β, IL-6, IL-8, GRO-α, superoxides, and ECP (Wong et al., 2007). This study provides a possible link between microbe-induced innate immunity and the exacerbation of asthma through activation of eosinophils.

Advances in the understanding of the eosinophil's functions in the pathogenesis of asthma have suggested that therapies targeting eosinophil regulators (humanized anti-IL-5 and CCR3 antagonists) could be promising for asthma patients (Hogan et al., 2008).

1.1.3.3. T lymphocytes

T lymphocytes constitute the majority of lung lymphocytes in normal individuals and are located within the airway, alveolar epithelium, and interstitium (Baraldo et al., 2007). Naïve T helper cells require two signals for activation. The first signal orginates from the interaction between the T-cell receptor (TCR) and peptide antigen-class II MHC presented on APCs, and is followed immediately by the second signals by the interaction between costimulatory molecules such as CD28, B7, OX40, OX40L, etc. Then, activated T helper cells begin to divide into a specific clone of effector cells, comprising of Th1, Th2, regulatory T (Treg), and Th17, depending on their distinct cytokine-secretion phenotype and unique functions (Kaiko et al., 2008). In particular, Th2 cells, which secrete IL-4, IL-5, IL-9, and IL-13 and activate B cells, play a key role in the development of asthma (Georas et al., 2005).

There are a broad array of mechanisms involved in Th2 polarization. When IL-4 binds to its receptor on the surface of T cells, the receptor subunits move together and Janus kinase (JAK)1, and JAK3 are activated, leading to the dimerization and translocation of STAT-6 (Kaiko et al., 2008). In the nucleus,

STAT-6 activates the expression of zinc finger transcription factor GATA-3, which is necessary for Th2 polarization (Kaplan et al., 1996; Zheng and Flavell, 1997). GATA-3 increases transcription of Th2 cytokines genes, selectively differentiates Th2 cells, and inhibits T-bet, a critical transcription factor for Th1 cells (Zhu et al., 2006). In, addition, other transcription factors are also associated with Th2 differentiation. It has been shown that NF-κB regulates Th2 polarization by controlling the expression of GATA-3 (Das et al., 2001). c-MAF (musculoaponeurotic fibrosarcoma oncogene homolog), specific to Th2 cells, is responsible for the regulation of IL-4 synthesis (Ho et al., 1998). Nuclear factor of activated T-cells (NFAT) and activator protein-1 (AP-1) synergize to promote the expression of IL-4 (Georas et al., 2005). In addition to IL-4, IL-6 and IL-11 could induce Th2 cells polarization by stimulating the production of IL-4 and inhibiting the secretion of interferon (IFN)γ (Curti et al., 2001; Dodge et al., 2003). Furthermore, the inducible costimulatory protein (ICOS), a member of the CD28 family, has been found to mediate Th2 responses by the enhancement of IL-4 receptor signaling (Watanabe et al., 2005). The CD28 ligand can induce GATA-3 and promote Th2 cells polarizatoin independent of IL-4 (Rodriguez-Palmero et al., 1999).

Th2 cells produce IL-4, IL-5, IL-9, and IL-13, contributing to the characteristic features of asthma (Baraldo et al., 2007). IL-4 is essential for driving the differentiation of naïve Th0 cells into Th2 cells (Kopf et al., 1993). IL-

4 is also required for the activation and differentiation of B cells, and subsequently synthesis and secretion of IgE and IgG4 (IgG1 in the mouse) (Bonnefoy et al., 1996). In addition, IL-4 has been shown to regulate extracellular matrix proteins and collagen, suggesting it plays roles in airway remodeling in asthma (Liu et al., 2002; Postlethwaite et al., 1992). IL-5 was originally identified for its role in activating B cells (Takatsu et al., 1994). Now IL-5 has been recognized as the major regulator in the maturation, differentiation, and activation of eosinophils (Rothenberg and Hogan, 2006). IL-9 has been shown to be involved in airway inflammation, mucus hypersecretion, and AHR in asthma (Cheng et al., 2002). IL-9 can also act on other cells such T cells, B cells and mast cells during the allergic responses (Soussi-Gounni et al., 2001). IL-13 is the most important Th2 effector cytokine, contributing to almost all characteristic features of asthma independent of other Th2 cytokines (Wills-Karp, 2004). Although the exact mechanisms by which IL-13 induces allergic reponses remain unknown, numerous studies have suggested the importance of IL-13 in the effector phase of asthma by regulating eosinophilic inflammation, isotype class swiching in B cells to IgE synthesis, induction of chemokines and adhesion molecules, subepithelial fibrosis, mucus hypersecretion, and AHR (Wills-Karp and Chiaramonte, 2003).

In recent years, many distinct Treg cells, including CD8+ Treg cells, natural killer (NK) T cells, and several different CD4+ Treg cells, have been identified as key players in immune tolerance (van Oosterhout and Bloksma, 2005). Of these

Treg cells, naturally occurring CD4+CD25+ Treg cells and IL-10-secreting CD4+ Treg cells have been shown to suppress the Th2 responses to allergen (Larche, 2007). IL-10, the main cytokine secreted by these Treg cells, inhibits the activation of inflammatory cells including mast cells, eosinophils, APCs, and Th2 cells. In addition, IL-10 enhances Ig isotype switching in B cells, which subsequently augment the IL-10-secreting CD4+ Treg cells (Hawrylowicz and O'Garra, 2005). Furthermore, the level of IL-10 has been shown to be related to the anti-inflammatory effects of glucocorticoid (Hawrylowicz and O'Garra, 2005).

1.1.3.4. B lymphocytes

B lymphocytes originate from bone marrow, and then enter peripheral lymphoid organs where they differentiate through several transitional stages and eventually become mature B lymphocytes (Larosa and Orange, 2008). Terminally differentiated B lymphocytes are known as plasma cells which are essential for the production of a secreted Ig (Matthias and Rolink, 2005). A variety of nuclear factors such as E-box factors, early B-cell factors, and NF-κB, are involved in the development and functions of B lymphocytes (Matthias and Rolink, 2005). Especially, several stages of late B lymphocyte differentiation and maturation are influenced by some components of NF-κB pathway. For example, p50-deficient mice lack marginal zone B cells and c-Rel (v-rel reticuloendotheliosis viral oncogene homolog) deficient mice show reduced numbers of marginal zone B

cells (Cariappa et al., 2000). Furthermore, p50/p52 double knockout mice fail to generate mature B cells (Franzoso et al., 1997).

B lymphocytes play an important role in the pathogenesis of asthma through production of Ig (Barnes, 2008). Under the influence of CD40 ligand and cytokines, mainly IL-4 and IL-13, B cells undergo immunoglobulin class switching to IgE. Following challenge by allergens, crosslinking of allergen-IgE complex with FcεRI on the surface of mast cells leads to mast cell degranulation and synthesis of lipid mediators, known as the early phase of the allergic response. IgE also binds to low affinity IgE receptor (FcεRII) expressed on other inflammatory cells, such as B cells, macrophages, and eosinophils, thereby augmenting allergic reactions. Furthermore, allergen-IgE complex binds to CD23, expressed by activated B cells, enhancing antigen presentation to T cells (Carlsson et al., 2007). Anti-IgE monoclonal antibody, omalizumab, has been shown to reduce airway inflammation and exacerbations in asthma patients (Avila, 2007). Besides the production of Ig, studies have suggested that B cells with B7 costimulatory molecules might be necessary for the development of CD4+ effector cells in a polarized Th2 response in animal models (Liu et al., 2007).

1.1.3.5. Epithelial cells

Epithelial cells are essential in host defense and inflammation, bridging innate immune responses and adaptive immune responses including DC, T cells, and B cells in the pathogenesis of asthma (Figure 1.2) (Schleimer et al., 2007).

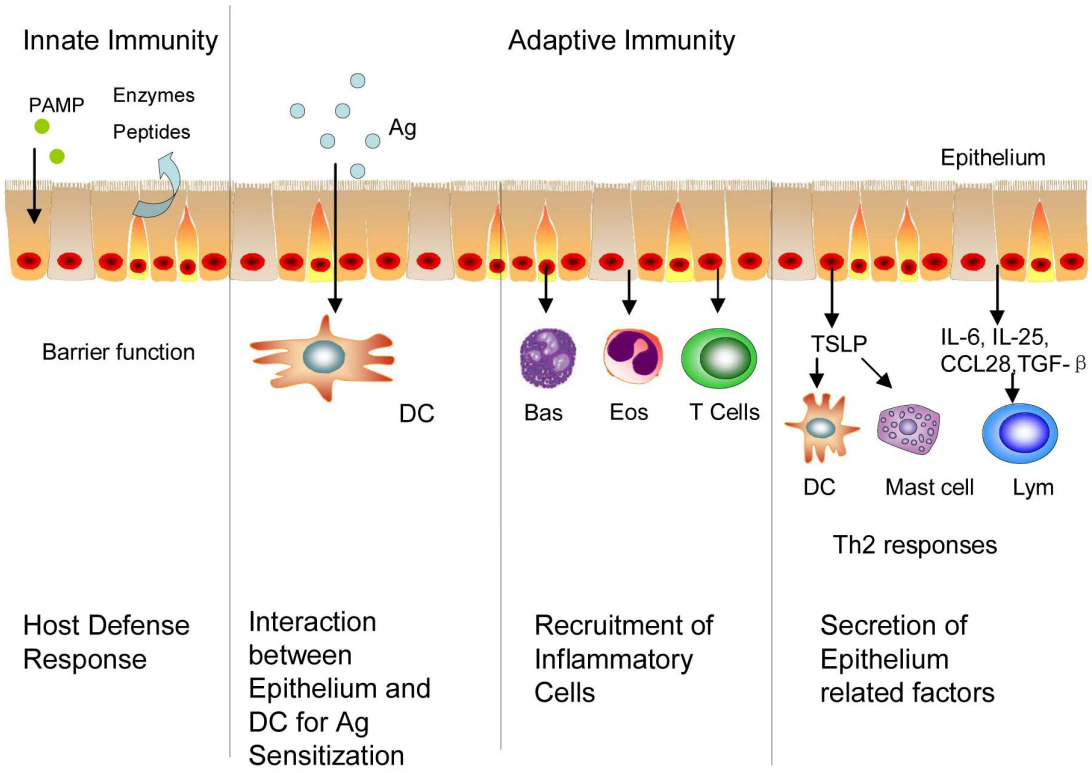


Figure 1.2 Roles of epithelium on innate and adaptive immunity (Adapted from Schleimer et al., 2007). Definition of abbreviations: TSLP = thymic stromal lymphopoietin; DC = dendritic cell; Bas = basophil; Eos = eosinophil; PAMP = pathogen-associated molecular pattern; Ag = antigen.

The role of epithelium in innate immune response has been known for decades. Epithelium protects the airways from most microorganisms via secretion of numerous molecules including enzymes, peptides, protease inhibitors, various small molecules, etc (Schleimer et al., 2007). Epithelium serves as the main barrier for the airways; abnormal barrier function of the epithelium has been linked to high incidences of asthma, suggesting the role of epithelium dysfunction in asthma (Hudson, 2006).

Besides being involved in innate immunity, epithelial cells play an even more important role in adaptive immunity, orchestrating asthmatic responses. Enzymatically active allergens stimulate epithelial cells, leading to the activation of NF-κB pathway, and production of chemokines (i.e. CCL17 and CCL20) and cytokines (i.e. GM-CSF, IL-6, IL-25, and thymic stromal lymphopoietin [TSLP]), which subsequently attract and activate DC, producing Th2 cells skewed responses (Hammad and Lambrecht, 2008). TSLP, a IL-7-like cytokine produced by epithelial cells in the airways, plays a central role in driving DC-mediated Th2 cell response (Allakhverdi et al., 2007). Upon stimulation of epithelial cells by TLR ligands or IL-4, TSLP is released and interacts with DC, causing the upregulation of costimulatory molecules such as CD40, OX40, and CD80, and Th2 polarization (Holgate, 2007; Kato et al., 2007).In addition, TSLP has been shown to directly activate mast cells and cause the subsequent secretion of cytokines such as IL-5, IL-6, IL-13, and GM-CSF (Allakhverdi et al., 2007).

Furthermore, the expression of TSLP is increased in the asthmatic airways and correlates with the severity of asthma (Ying et al., 2005). Besides DC and T cells, epithelial cells also produce several molecules, such as CCL28, IL-6, and TGF-β, which regulate the activation, differentiation, and migration of B cells (Schleimer et al., 2007). In addition, it has been shown that the NF-κB pathway airway in epithelial cells is essential for the lung inflammation in response to local or systemic stimuli, in transgenic mice express a constitutively active form of IKKβ under control of the epithelial-specific CC10 promoter (Cheng et al., 2007).

1.1.3.6. Mucus hypersecretion

Different phenotypes of airway muscus hypersecretion, including luminal mucus overproduction, goblet cell hyperplasia, submucosal gland hypertrophy, and plasma exudation, are characteristic pathological features of asthma (Rogers, 2004). Airway mucus contains about 2% mucin, a high-molecular-weight glycoprotein, which is secreted by goblet cells in epithelial and mucous cells in the submucosal glands (Finkbeiner, 1999; Rogers, 2003). Of the 19 human mucin genes identified to date, Muc5ac, Muc5b, and Muc2 gene products have been considered the major gel-forming mucins of the airways (Morcillo and Cortijo, 2006).

A wide array of exogenously inhaled allergens and endogenous mediators can induce mucus hypersecretion (Morcillo and Cortijo, 2006). Cumulative evidence has suggested that IL-13, which independently induces goblet cell hyperplasia

without IL-4 and IL-5, is probably the key regulator of mucus hypersecretion (Wills-Karp and Chiaramonte, 2003). IL-9, another Th2 cytokine, could also enhance mucus secretion. In addition, oxidant stresses are associated to increased mucin secretion. NF-E2-related factor-2 (Nrf2) is known to plays a crucial role in the regulation of many antioxidant genes. Nrf2 deficient asthmatic mice displayed amplified inflammatory responses including mucus cell hyperplasia (Rangasamy et al., 2005). Moreover, MMP-9, an asthma related protease, is involved in the elevation of Muc5ac expression via stimulation of epidermal growth factor receptor in human airway epithelial cells (Deshmukh et al., 2005; Ohbayashi and Shimokata, 2005).

NF-κB pathway also plays an important role in mucus hyperserection in mouse asthmatic models (Desmet et al., 2004; Poynter et al., 2004). It has been reported that activated NF-κB could bind to the κB site in the 5`-flanking region of the Muc2 gene in of epithelial cells, leading to the transcription of Muc2 mucin (Li et al., 1998). In addition, tumor necrosis factor-α (TNF-α) promotes the transcription of Muc5ac and its products in airway epithelium through a NF-κB (IKK)β dependent mechanism (Lora et al., 2005). The contribution of NF-κB pathway to mucus production is further supported by a mouse asthma model with IKKβ deletion in the airway (Broide et al., 2005).

1.1.3.7. Airway hyperresponsiveness

AHR is a characteristic feature of asthma, although mechanisms of AHR in asthma remain unclear (Busse and Lemanske, 2001). There are at least two different components of AHR: the variable and persistant components (Cockcroft and Davis, 2006). The variable component of AHR is probably related to airway inflammation, reflecting the acute effects of airway inflammation, whereas the persistent component of AHR is likely associated with structural changes of the airways, known as airway remodeling, reflecting the chronic effects of airway inflammation (Cockcroft and Davis, 2006). However, AHR is such a complicated process that it is almost impossible to simply arbitrarily separate it into several distinct components.

AHR is closely related to the inflammatory process of asthma. Upon activation by antigens, Th2 cells produce cytokines, including IL-4, IL-5, IL-9, and IL-13, which orchestrate the recruitment and activation of other inflammatory cells, such as mast cells and eosinophils, which contribute to the initiation of AHR (Wills-Karp, 1999). Blockade of IL-4 receptor has been shown to inhibit the allergen-induced AHR in mice and inhaled IL-4 induced AHR in response to methacholine in a human placebo-controlled study, indicating that IL-4 can increase AHR in asthma (Gavett et al., 1997; Shi et al., 1998). IL-5 is responsible for the differentiation, maturation, and activation of eosinophils, and plays a critical role in AHR by mobilizing and activating esoinophils, leading to the release of pro-inflammatory products such as major basic protein and cysteinyl-

leukotrienes, which are closely associated with AHR (Rothenberg and Hogan, 2006). IL-13, the major effector Th2 cytokine in asthma, can independently induce AHR in the absence of lymphocytes, mast cells, IL-4, and IL-5 (Grunig et al., 1998; Wills-Karp, 2004; Wills-Karp et al., 1998; Yang et al., 2001). Moreover, IL-13 may induce the contraction and proliferation of ASM cells, directly via IL-4 receptor α chain or IL-13 receptor α1 and α2 chain expressed on ASM cells (Laporte et al., 2001; Moore et al., 2002), or indirectly via the stimulation of cysteinyl-leukotriene (Espinosa et al., 2003; Vargaftig and Singer, 2003) . Mast cells release mediators such as histamine, LTs, PAF, which cause microvascular leakage, increase mucus production, and induce bronchoconstriction (Wills-Karp, 1999). Furthermore, mast cells contribute to airway remodeling, through direct interaction with ASM cells and the release of mediators such as tryptase and cytokines which regulate the ASM cell function and induce goblet cell hyperplasia (Okayama et al., 2007). Moreover, eosinophils produce basic proteins such as ECP and MBP which damage the epithelium of the airway, subsequently leading to AHR (Wills-Karp, 1999).

Besides inflammation, airway remodeling, generally described as structural changes in the airway such as epithelial metaplasia, airway fibrosis, and ASM hyperplasia, also contribute to AHR (Fixman et al., 2007). Both inflammatory cells and structural cells, such as epithelial cells, ASM cells, and fibroblasts, take part in the regulation of airway remodeling and AHR (Leigh et al., 2004).

Abundant data has shown that both the hyperplasia of ASM and enhanced deposition of extracellular matrix (ECM) are apparent in both asthmatic patients and animal models (Fixman et al., 2007; Woodruff et al., 2004). Quick reaction to inhaled allergens and rapid turnover rate of epithelium are also features of asthma. Furthermore, airway epithelium participates in airway remodeling by producting epithelial cell-specific growth factors and regulating goblet cell differentiation (Fixman et al., 2007). In addition, in asthmatic models with specific deletion of IKKβ in the epithelium, mice exhibited reduced mucus production, fibrosis, and airway thicking, suggesting NF-κB signaling pathways play a pivotal role in airway epithelium induced airway remodeling (Broide et al., 2005). Consistent with previous studies, recently, it has been shown that selective activation of NF-κB in the airway epithelium is sufficient to provoke AHR and cause the thickening of ASM (Pantano et al., 2008).

AHR, as a constant feature of the asthma, is the result of a combination of various mechanisms. Multiple measures should be done to reduce this prevalent and common phenotype in asthma patients.

1.1.4. Current therapy for asthma

Current therapy for asthma comprises of bronchodilators and anti-inflammatory agents. A combination of inhaled corticosteroids and short-acting or long-acting β2-adrenoceptor agonists is the first line therapy for the control of mild to severe asthma patients (Holgate and Polosa, 2008). Although most of the

asthmatics can be controlled by current therapy, up to 15% patients do not respond well to current therapy and suffer from uncontrollable symptoms (Amon et al., 2006). There is a demand to develop new drugs for asthma patients.

Inhaled corticosteroids (CS) are the most effective drugs for both the control of symptoms and improvement of lung function in asthma patients (Barnes, 2006). CS is able to quickly cross cell membranes and bind to glucocorticoid receptors (GR) (Rhen and Cidlowski, 2005). Following ligand binding, GR dimer translocates into the nucleus where CS regulates the transcriptional activity of target genes by different mechanisms including gene transactivation and gene transrepression, leading to the inhibition of expression of cytokines, chemokines and adhesion molecules (Goulding, 2004; Holgate and Polosa, 2008; Rhen and Cidlowski, 2005). Although CS targets a variety of inflammatory mechanisms and is highly effective for the control of asthma, 10 to 15% of patients are insensitive, even to high doses of oral corticosteroids. Some severe asthma patients need oral steroid as a regular treatment. Furthermore, there has been increasing concerns about the systemic and local side effects of CS. Reasonable add-on therapies may give greater benefits for asthmatic patients than increasing the dose of CS.

Selective β2 adrenergic agonists, the most important bronchodilator in asthma treatment, include short-acting (e.g. salbutamol and levalbuterol) and long-acting (e.g. salmeterol and formoterol) compounds (Amon et al., 2006). They act on β2 adrenergic receptors and result in the formation of cyclic adenosine

monophosphate through activation of G proteins, causing relaxation of smooth muscles of airways. However, recently, there are more and more studies suggesting that regular treatment with long-acting β2 adrenergic agonists could increase both exacerbation and death rates of asthma patients (Martinez, 2005; Salpeter et al., 2006). Although mechanisms regarding the deterioration of asthma symptoms through the usage of β2 adrenergic agonists remain unclear, asthma guidelines have suggested that long-acting β2 adrenergic agonists should be given in combination with CS (Kelly, 2007).

Leukotrienes, including cysteinyl LTs (LTC4, LTD4, and LTE4) and LTB4, are arachidonic acid derivatives formed by 5-lipoxygenase and 5-lipoxygenase-activating protein (Peters-Golden and Henderson, 2007). Cysteinyl LTs are the most potent endogenous bronchoconstrictors and play an important role in asthmatic inflammation. LTB4 is a potent chemoattractant for neutrophils, but has little effect on bronchoconstriction. Commonly used cysteinyl LT antagonists consist of montelukast and zafirlukast, which block cysteinyl LT1 receptor binding sites, thereby improving asthmatic symptoms. Furthermore, zileuton, a 5-lipoxygenase inhibitor, blocks endogenous production of both cysteinyl LTs and LTB4. It has been well documented that LT antagonists could clinically alleviate asthma symptoms and reduce the dose of CS in both adults and children (Dahlen, 2006).

IgE which recognizes allergens, is associated with the AHR and local inflammation in asthma. Omalizumab (Xolair), a recombinant DNA-derived humanized IgG1κ monoclonal antibody against IgE, was approved by the U.S. Food and Drug administration for the treatment of severe or refractory asthma patients in June 2003 (Avila, 2007). However, there are still lots of concerns about the side effects of omalizumab. There is insufficient information on the long-term effectiveness and side effects of this drug. Although the IgE pathway is not an important mechanism with which the immune system fights against the development of cancers, there was a small increase in the incidence of malignancies in omalizumab-treated groups compared with placebo-treated groups (Avila, 2007). The neoplasias caused by omalizumab are still being evaluated in Phase Ⅳ studies.

Theophylline is a moderately potent bronchodilator and has both immunomodulatory and anti-inflammatory actions at low concentration. Traditionally, the therapeutic effects of theophylline have been attributed to its nonselective phosphodiesterase inhibition, but there might be other mechanisms for this drug. The major limitation of theophylline is its narrow therapeutic index. Therefore, theophylline is now considered as the second-line therapy for asthma (Boswell-Smith et al., 2006).

1.1.5. New therapy for asthma

With an improved understanding of the pathophysiology of asthma, a variety of new approaches and new targets have been investigated in the last decade, providing insights for asthma therapies (Amon et al., 2006; Tarantini et al., 2007).

Th2 cytokines (e.g. IL-4, IL-5, and IL-13) are important in the development of asthma and are promising targets for the treatment. Efficacy of soluble IL-4 receptor (sIL-4R) has been investigated in two studies, both of which indicated sIL-R improves both lung function and asthma symptoms in patients (O'Byrne, 2006). However, there are no published reports confirming these results in larger scale trials. Anti-IL-5 humanized monoclonal antibodies (e.g. mepolizumab and SCH 557700) have been shown to decrease both blood and sputum eosinophils, but could not improve patient`s symptoms dramatically (O'Byrne, 2006).

TNF-α has been implicated in airway inflammation, AHR, and airway remodeling in asthma patients, especially severe refractory patients (Berry et al., 2006). Several TNF-α blockers, such as infliximab (a chimeric mouse/humanized mAb), etanercept (a soluble fusion protein combining 2p75 TNF receptors with an Fc fragment of human IgG1), and adalimumab (a fully human mAb) have been tested in clinical trials (Brightling et al., 2007). However, preliminary data from clinical trials are controversial. It is likely that TNF-α blockers would only work in a small group of severe refractory asthma patients. Heterogeneity in response to TNF-α blockers in asthma patients should be considered for future studies.

Phosphodiesterase type 4 (PDE4) is a group of enzymes encoded by at least four distinct genes (PDEA, PDEB, PDEC, PDED), which hydrolyze cAMP into 5-monophosphates, an inactive form (Fan Chung, 2006). PDE4 inhibitors act by increasing intracellular cAMP and have been shown to exert a wide range of anti-inflammatory effects (Boswell-Smith et al., 2006; Fan Chung, 2006). Several PDE4 inhibitors have been developed for the treatment of both asthma and COPD, but only Roflumilast (Altana) and Cilomilast (GlaxoSmithKline) have proceed to phase III clinical trials (Lipworth, 2005). There are doubts about the therapeutic values of PDE4 inhibitors and concerns about their side effects, particularly nausea and vomiting.

Protein kinases are a group of enzymes which add the γ phosphate of ATP or GTP to tyrosine, serine, and threonine residues of target proteins. Because kinases are critical in the amplification of inflammatory signals in the pathogenesis of asthma, several kinase inhibitors have been developed to reduce the allergic responses (Adcock et al., 2006). MAPK kinase inhibitor (U0126), p38 MAPK inhibitors (SB239063 and respirable p38alpha MAPK antisense oligonucleotide) and c-Jun NH2-terminal kinase (JNK) inhibitor (SP600125) have been shown to be able to attenuate allergic inflammation in asthmatic models (Duan and Wong, 2006). IKKβ inhibitors are associated with reduced airway inflammation and AHR in mouse model (Ziegelbauer et al., 2005). In addition, some tyrosine kinase inhibitors, such as Syk inhibitor (BAY 61-3606), Lyn inhibitor (Lyn-binding

peptide), and JAK3 inhibitor (CP-690550) are effective in asthmatic models (Kudlacz et al., 2008; Wong, 2005). Kinase inhibitors, especially those aiming at MAPK and NF-κB pathways, are promising as an accurate bullet targeting inflammatory cascades; however, specificity, efficacy, and possible side effects should be kept in mind, as these inhibitors proceed to clinical trials (Bain et al., 2003; Park and Christman, 2006).

1.2. NF-κB signaling pathway

1.2.1. Introduction of the NF-κB pathway

The nuclear factor-κB (NF-κB)/REL (v-rel reticuloendotheliosis viral oncogene homolog) family is a central player in coordinating both innate and adaptive immunity. Approximately 20 years ago, three proteins (NF-κB, v-Rel, and Dorsal) were coincidently discovered and soon demonstrated to be different members of the same NF-κB family (Sen and Baltimore, 1986; Singh et al., 1986). Today, with an exponential increase in information regarding NF-κB pathways, it is now considered a ubiquitous pathway involved in the regulation of a broad array of genes in response to diverse stimuli (Perkins, 2007).

The NF-κB/REL transcription factor family consists of two subfamilies: the NF-κB proteins and the Rel proteins (Figure 1.3) (Gilmore, 2006; Perkins, 2007). All of these proteins share a structurally conserved DNA-binding/dimerization domain, known as Rel homology domain (RHD), and have functions conserved from fly drosophila melanogaster to humans.

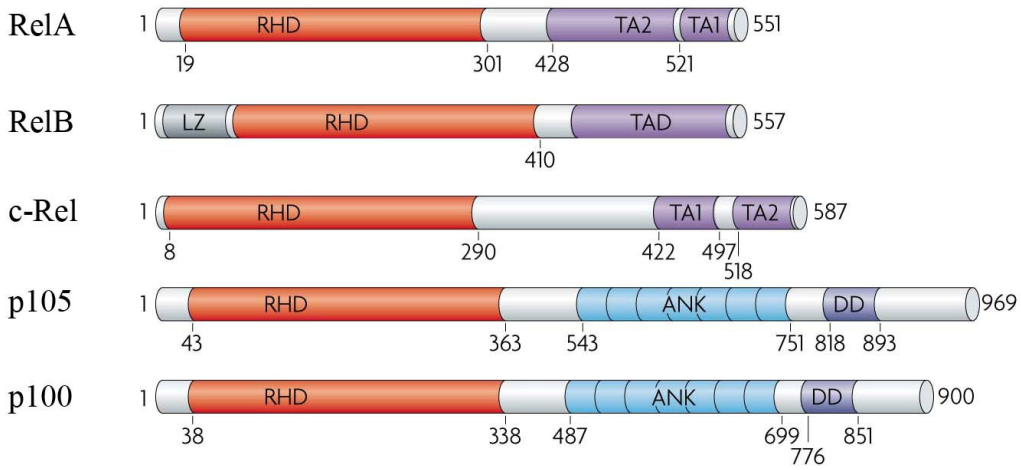


Figure 1.3 Members of NF-κB family (Adapted from Perkins, 2007). Definition of abbreviations: ANK = ankyrin-repeat motifs; DD = death domain; RHD = Rel homology domain; TA/TAD = transcriptional activation domain.

The NF-κB proteins, including p105 and p100, contain long C-terminal domains with several ankyrin repeats which auto-inhibit these proteins. Upon either limited proteolysis or arrested translation, NF-κB become shorter proteins (p105 to p50 and p100 to p52), which are the main DNA-binding units when they form dimers with other Rel proteins. The Rel proteins including c-Rel, RelB, and RelA (p65), contain C-terminal transactivation domains which activate transcription of genes in dimeric forms. Furthermore, the p50/p65 heterodimer, the most common activated form of NF-κB transcription factor, is ubiquitous in all cell types; p52 is expressed in epithelium, macrophages, lymphocytes, and dendritic cells; RelB is restricted to dendritic cells and lymphocytes; c-Rel is confined to haematopoietic cells (Bottero et al., 2006). The specific expression of NF-κB proteins in particular cell types implies their distinct functions in immune system.

Besides NF-κB family, there are also two other important groups of proteins (IκB family and IKK complex) which are involved in the regulation of NF-κB pathways. There are three principal IκBs (IκBα, IκBβ, and IκBε) which retain NF-κB dimers in the cytoplasm, in part by masking a conserved nuclear localization sequence in the RHD of NF-κB subunits (Li and Verma, 2002). IKK complex comprises of three main subunits: the catalytic subunits IKKα and IKKβ (also called IKK1 and IKK2 repectively), and the regulatory subunit IKKγ (also called NF-κB essential modifier [NEMO]) (Hacker and Karin, 2006; Scheidereit, 2006). IKKβ and NEMO are the predominant components in IKK complex in the

canonical pathway, whereas IKKα appears to stimulate p100 precursor processing and regulates NF-κB-independent developmental processes (Bonizzi and Karin, 2004; Scheidereit, 2006). Various genetic experiments in mice have been done to investigate the functions of NF-κB, IκB, and IKK in physiological processes and human diseases (Table 1.2) (Gerondakis et al., 2006; Pasparakis et al., 2006). In most quiescent cells, NF-κB forms an inactive complex with its inhibitor, IκBα, in the cytoplasm. Upon various stimuli, such as pro-inflammatory cytokines (TNF-α and IL-1), lipopolysaccharides (LPS), engagement of TCR, viruses (rhinovirus and adenovirus), oxidative stress (O_3, H_2O_2 and NO_2), and ultraviolet light, NF-κB pathways will be activated predominantly through canonical and non-canonical pathways (Figure 1.4) (Perkins, 2007). In the canonical pathway, numerous NF-κB stimuli, such as TNF-α, antigen, LPS, can activate the IKK complex. The regulatory subunit NEMO is essential to the activation of IKK complex. In response to stimuli, NEMO is ubiquitylated at Lys^{63} and subsequently recruits some kinases such as transforming growth factorβ activated kinase-1, which phosphorylate IKKβ at $Ser^{177/181}$, leading to the activation of IKK complex. IκBα is phosphorylated by the activated IKK complex at Ser^{32} and Ser^{36}. Then, phosphorylated IκBα is ubiquitinylated at Lys^{21} and Lys^{22} by β-transducin repeat-containing protein (β-TRCP), and subsequently degraded by the 26S proteasome.

Table 1.2 Knockout mouse models for the NF-κB pathway (Adapted from Gerondakis et al., 2006).

Mutated Gene	Phenotype in knockout mice	References
RelA (P65)	Die at E15 to E16; TNF-α mediated fetal hepatocyte apoptosis	(Beg et al., 1995b)
RelB	Multiple organ inflammations and hematopoietic abnormalities	(Burkly et al., 1995);
c-Rel	Defects in lymphocyte and macrophage functions	(Grumont et al., 1998)
NF-κB1 (p105/p50)	Defects in lymphocyte activation	(Sha et al., 1995)
NF-κB2 (p100/p52)	Defects in lymphoid organ development	(Franzoso et al., 1998)
IκBα	Die postnatally due to immune deficiency; constitutive NF-κB acitivity	(Beg et al., 1995a)
IκBε	Increased expression of certain Ig isotypes and cytokines	(Memet et al., 1999)
IKKα (IKK1)	Die at birth; Skeletal and epidermal defects	(Hu et al., 1999)
IKKβ (IKK2)	Die at E13 due to TNF-α dependent liver apoptosis	(Li et al., 1999)
IKKγ (NEMO)	Die at ~E10 due to hepatocyte apoptosis	(Kim et al., 2003)

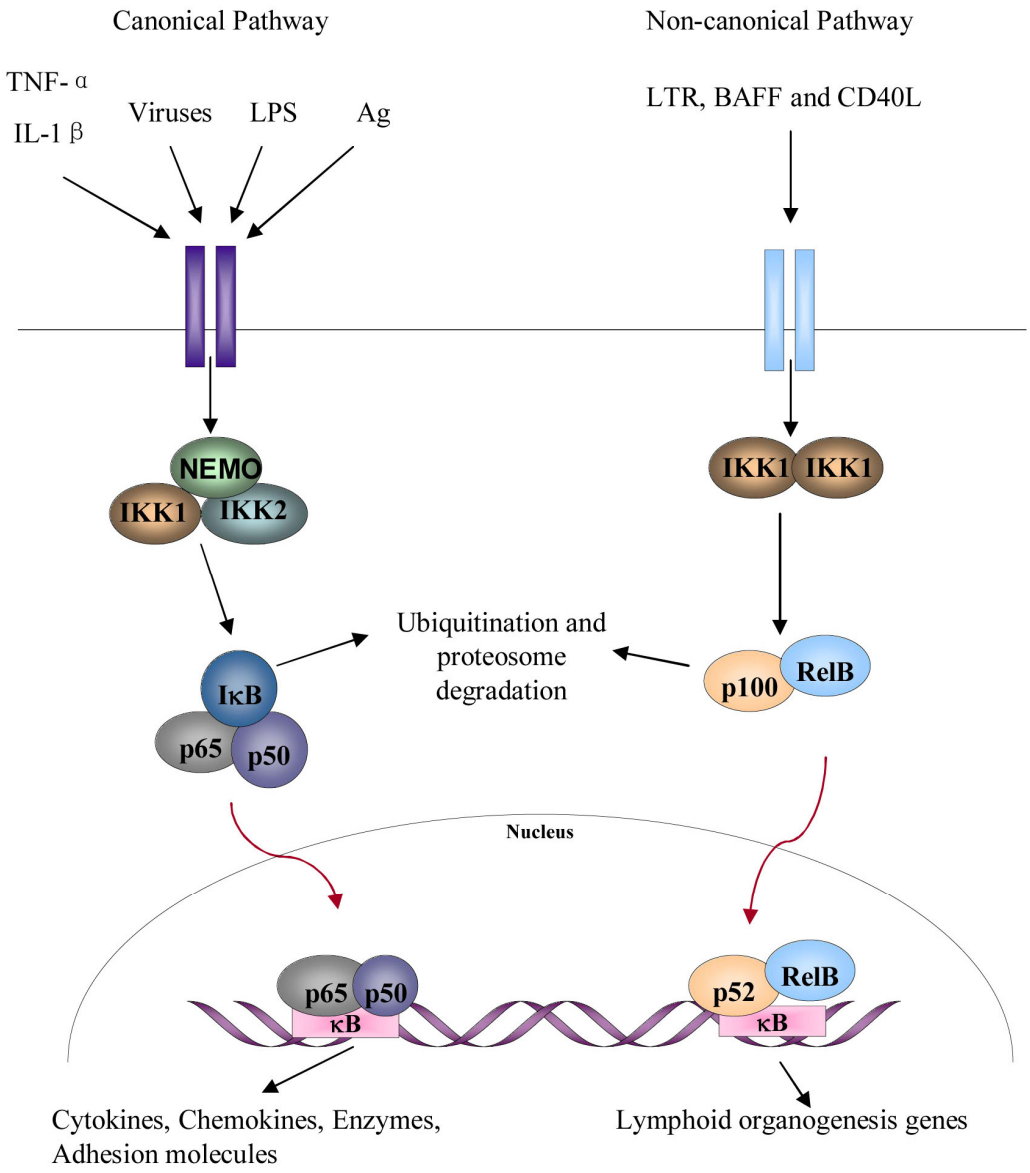


Figure 1.4 Canonical and non-canonical pathways of the NF-κB family.

After that, NF-κB dimer is released from the cytoplasmic NF-κB-IκB complex and translocates to the nucleus. In the nucleus, the NF-κB dimer binds to a 9-10 base pair DNA site, known as κB sites (5'-GGGPuNPyPyPyCC-3', where Pu = purine, N = any nucleic acid, Py = pyrimidine), leading to the transcription of a variety of downstream genes. These genes comprise of cytokines (TNF-α, IL-1β, IL-5, IL-6, IL-13 and GM-CSF), chemokines (IL-8, eotaxin, RANTES, and monocyte chemoattractant protein-1 [MCP-1]), adhesion molecules (ICAM-1, VCAM-1, and E-selectin), and enzymes (COX-2 and iNOS) (Bonizzi and Karin, 2004). In the non-canonical pathway, the p100-RelB dimer is activated by phosphorylation of the C-terminal region of p100 by the IKKα homodimer. Then, the proteasome-dependent processing of p100 to p52 causes the activation and translocation of p52-RelB heterodimers. After that, the binding of p52-RelB to distinct κB sites in the nucleus results in the expression of genes mainly involved in the development and maintenance of secondary lymphoid organs (Bonizzi and Karin, 2004). However, recent studies have suggested that the regulation of NF-κB pathways is more complicated than that defined by the canonical and non-canonical pathways. For instance, there are many post-translational modifications for NF-κB subunits. RelA is phosphorylated by protein kinase A at Ser^{276}, by glycogen synthase kinase-3β (GSK-3β) at Ser^{468} and Ser^{536}, and by IKKβ at Ser^{536} (Mattioli et al., 2004; Perkins, 2007; Yang et al., 2003). Moreover, the transcriptional activity of RelA is regulated by both coactivators, such as p300 and

CREB binding protein (CBP), and corepressors such as histone deacetylase (HDAC)s (Li and Verma, 2002). Furthermore, there are crosstalks between NF-κB pathways and other signaling pathways including JNK, p53, and various nuclear receptors (Perkins, 2007).

NF-κB pathways play a central role in both biological functions and human diseases. Genetic studies have unequivocally revealed a pivotal role of NF-κB in the differentiation and maturation of hematopoietic cells and lymphoid organogenesis (Figure1.5) (Bottero et al., 2006). Due to the rapid turnover rate of hematopoietic cells, the immune system including all lymphoid and myeloid lineages, is heavily influenced by NF-κB pathways. Aberrant activation of NF-κB pathways have been linked to a variety of diseases such as inflammation, cancer, arthritis, atherogenesis, asthma, etc (Kumar et al., 2004). However, further studies have revealed more complicated roles for NF-κB pathways (Greten et al., 2007; Perkins and Gilmore, 2006). NF-κB pathways can exhibit opposing behaviors in the same biological process. For instance, NF-κB promotes cancer pathogenesis through induction of anti-apoptotic genes and proinflammatory genes, whereas NF-κB can also suppress tumor activity by induction of p53-induced apoptosis and pro-apoptotic genes (Perkins and Gilmore, 2006). It has been shown that the inhibition of IKKβ could increase the secretion of IL-1β in an endotoxin-induced sepsis mouse model, via a caspase dependent manner, suggesting potential complications for the long-term inhibition of NF-κB pathway (Greten et al., 2007).

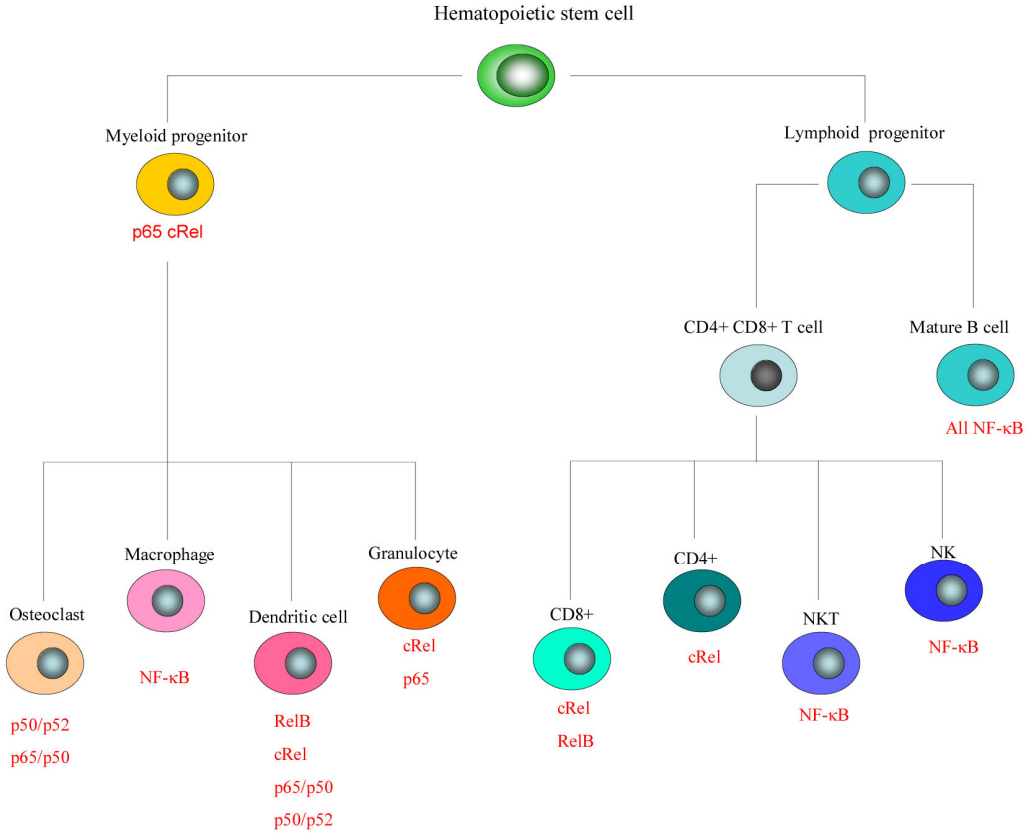


Figure 1.5 The functions of NF-κB in hematopoietic differentiation (Adapted from Bottero et al., 2006). All NF-κB refers to p65, RelB, cRel, p50, and p52.

Further studies in this area are required for better understanding the complex mechanisms of NF-κB activity and related diseases.

1.2.2. Role of the NF-κB pathway in allergic inflammation

Cumulative evidence has suggested that the NF-κB signaling cascade plays a pivotal role in the initiation and development of asthma in both allergic animal models and human asthma patients (Kumar et al., 2004; Poynter et al., 2002). Persistent NF-κB DNA-binding activity was detected in both bronchial biopsies and induced sputum of asthmatics. In addition increased levels of p65 were found in airway epithelial cells from bronchial biopsies of asthmatics (Hart et al., 1998). High levels of phosphorylated IκBα, IKKβ, p65, and NF-κB DNA-binding activity were detected in peripheral blood mononuclear cells (PBMC) of severe asthmatics compared to normal subjects (Gagliardo et al., 2003). Moreover, these PBMC produced high levels of IL-8, RANTES, and GM-CSF, which were inhibited by the NF-κB inhibitor (Gagliardo et al., 2003). House dust allergen Der p1, a common trigger of bronchial asthma, promotes NF-κB activation in the asthmatic bronchial epithelial cells (Stacey et al., 1997). Furthermore, a broad array of genes involved in pathogenesis of asthma, such as IL-9, IL-13, eotaxin, TSLP, ICAM-1, VCAM-1, E-selectin, etc, contain κB sites within their promoters, supporting the role of NF-κB in the allergic inflammation (Ahn and Aggarwal, 2005). NF-κB also promotes the transcription of genes that are involved in airway remodeling and mucus hypersecretion in the bronchial epithelium (Broide et al.,

2005). Several NF-κB knockout asthma mouse models have shown reduced airway inflammations following the challenge by allergens (Donovan et al., 1999; Poynter et al., 2004; Yang et al., 1998). Moreover, corticosteroids, the most effective drug for the control of asthma, are also a potent inhibitor of NF-κB pathways in mice and cultured cells (Auphan et al., 1995). In addition to corticosteroids, numerous other NF-κB inhibitors, such as NF-κB-specific decoy oligonucleotide, p65-specific antisense oligonucleotide, and IKKβ selective small molecule inhibitors have demonstrated beneficial effects in experimental asthma models (Birrell et al., 2005; Choi et al., 2004; Desmet et al., 2004; Ziegelbauer et al., 2005). Collectively, these findings suggest that the constitutive activation of NF-κB pathways is one of the primary effectors in the pathogenesis of asthma and pharmacological agents targeting the NF-κB cascade may be promising in the treatment of asthma.

1.3. Inhibitors of NF-κB signaling cascades

NF-κB plays a pivotal role in both physiological and pathological processes, regulating immune responses, the development of cells, and even the expression of some anti-viral genes. Thus, a variety of therapeutic approaches targeting the NF-κB pathway have been developed, especially for chronic inflammation and cancer where this pathway is often constitutively active (Ahn et al., 2007; D'Acquisto et al., 2002). With an increasing understanding of the NF-κB pathway, more than 700 inhibitors, aimed at different levels of this pathway, have been

identified (Gilmore and Herscovitch, 2006). These compounds comprise of peptides, small RNA/DNA, natural medicinal herbs, and small synthetic molecules. However, until now, no compounds, designed as a specific NF-κB inhibitor, have been approved clinically, indicating that more work needs to been done in developing potent NF-κB inhibitors with minimal side effects (Uwe, 2008).

1.3.1. The GSK-3β inhibitor

1.3.1.1. The GSK-3 pathway

Glycogen synthase kinase-3 (GSK-3) is a serine-threonine kinase that exists in two isoforms in mammalian cells, namely GSK-3α and GSK-3β (Woodgett, 1990). Although it was originally discovered based on its ability to phosphorylate and inactivate glycogen synthase, GSK-3 was found to play a key role in regulating the functions of a wide array of substrates including metabolic and signaling proteins, structural proteins, and more importantly transcription factors (Embi et al., 1980; Jope and Johnson, 2004; Rylatt et al., 1980).

One of the best studied functions of GSK-3 is its role in the wingless and int-1 (WNT) signaling pathway. A proportion of GSK-3 forms a stable complex with axin, the adenomatous polyposis coli protein, and β-catenin in cells, leading to the phosphorylation of β-catenin and subsequent ubiquitination and proteolytic degradation. Upon binding of WNTs to its receptors, GSK-3 is inhibited by FRAT (frequently rearranged in advanced T-cell lymphomas) and dissociates from the

complex. Then, the dephosphorylated β-catenin translocates into the nucleus, binds to the T-cell factor transcription factor, and stimulates the expression of embryogenesis related genes (Cohen and Frame, 2001).

GSK-3 activity can be regulated by phosphorylation, localization, and interaction with GSK-3 binding proteins. Among these mechanisms, phosphorylation is the most studied one. GSK-3 activity is greatly inhibited following phosphorylation at a serine residue near its N-terminal (Ser^{21} in GSK-3α or Ser^{9} in GSK-3β). In contrast, GSK activity is enhanced by phosphorylation of Tyr^{279} in GSK-3α or Tyr^{216} in GSK-3β (Jope and Johnson, 2004). The activity of GSK-3 is also regulated by its substrate. GSK-3 is a unique protein kinase in that some of its substrates need to be first phosphorylated by another protein kinase at a serine or threonine residue, known as priming phosphate, which is located four residues carboxy-terminal to the site of GSK-3 phosphorylation (ter Haar et al., 2001). In active state, the substrate with a priming phosphate binds to a pocket in GSK-3 which contain three crucial basic residues (Arg^{96}, Arg^{180}, and Lys^{205}), aligning it in a position that GSK-3 can phosphorylate a serine or threonine four residues to the priming phosphate and activate the substrate (Cohen and Frame, 2001; Dajani et al., 2001).

1.3.1.2. GSK-3β and the NF-κB pathway

Cumulative evidence indicates that GSK-3 is necessary for the full transcriptional activity of NF-κB pathway (Dugo et al., 2007; Jope et al., 2007). In

year 2000, Hoeflich and collegues showed that GSK-3β knockout mice resulted in embryonic lethality and displayed a phenotype consistent with p65- and IKKβ-deficient mice (Hoeflich et al., 2000). In addition, GSK-3β is necessary for the NF-κB mediated anti-apoptotic response to TNF-α and the role of GSK-3β in this biologic process could not be compensated for by GSK-3α (Hoeflich et al., 2000). Followed by this discovery of the role of GSK-3β in the regulation of NF-κB pathway, a series of studies using GSK-3β inhibition reveals that GSK-3β can positively regulate the activity of NF-κB pathway (Dugo et al., 2005; Dugo et al., 2007; Martin et al., 2005; Whittle et al., 2006). It has been shown that inhibition of GSK-3 could suppress transcription of the NF-κB-dependent inducible nitric oxide synthase gene, and the inhibition of GSK-3 protects hepatocytes from TNF-α induced apoptosis through the phosphorylation of p65 (Schwabe and Brenner, 2002). It was reported that NF-κB activation upon stimulation by LPS, IL-1β, or cigarette smoke condensate was entirely inhibited in GSK-3$\beta^{(-/-)}$ cells (Takada et al., 2004). In addition, GSK-3β is necessary for the efficient translocation of p65 into nucleus and binding to the promoter regions of IL-6 and MCP-1 upon stimulation by TNF-α (Steinbrecher et al., 2005). It has been postulated that in response to TNF-α stimulation, GSK-3β facilitates the phosphorylation of NF-κB subunit p105, leading to the formation of NF-κB subunit p50 (Demarchi et al., 2003). Interestingly, several groups have reported that a high level of β-catenin following the inhibition of GSK-3β could reduce the activity the NF-κB through

certain unidentified mechanisms (Deng et al., 2002; Deng et al., 2004; Duan et al., 2007). Taken together, GSK-3β has been shown to positively regulate the activity of NF-κB pathway, albeit the fact that the exact mechanisms remain to be elucidated.

1.3.1.3. GSK-3β inhibitors

Due to the crucial role of GSK-3β in the pathophysiology of inflammatory disorders, there are a variety of GSK-3β inhibitors that have been tested as therapeutics for diabetes, Alzheimer`s disease, and inflammatory diseases (Meijer et al., 2004). Lithium, a popular drug for the bipolar disorder, is the best-characterized GSK-3 inhibitor and widely used as a pharmacological tool to investigate the role of this kinase in biological processes (Jope, 2003). However, lithium also affects other kinases and its Ki value (mM) is relatively high compared with other specific inhibitors (Bain et al., 2003; Doble and Woodgett, 2003). Insulin, an endogenous GSK-3 inhibitor, may also exert its beneficial effects in the treatment of sepsis by reducing the activity of GSK-3β (Dugo et al., 2006; Eldar-Finkelman et al., 1999). Besides, a panel of adenosine triphosphate (ATP)-competitive GSK-3 inhibitors, such as SB415286, beryllium, and hymenialdisine, has been identified by screening chemical databases (Doble and Woodgett, 2003). However, because there is only a single amino acid difference in the ATP-binding site of GSK-3 (Glu^{196} in GSK-3α, Asp^{133} in GSK-3β), these inhibitors are unable to distinguish between the two isoforms of GSK-3 (Meijer et

al., 2004). Since ATP-binding sites among all kinases are similar to some extent, these ATP-competitive GSK-3 inhibitors could affect other protein kinases. In addition this ATP-competitive inhibition is easily reversed by a high concentration of ATP, a constitutive component of cells.

In year of 2002, Martinez and colleagues described the first non-ATP-competitive GSK-3β inhibitor, known as 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) (Figure 1.6) (Martinez et al., 2002). TDZD-8 was shown to be a potent GSK-3β inhibitor (IC50 = 2 μM) and exhibited low specificity (IC50 > 100 μM) for cyclin-dependent kinases, enzymes structurally similar to GSK-3β. In addition, TDZD-8 did not show inhibitory effects on other kinases such as protein kinase A (PKA), protein kinase C (PKC), and casein kinase II (CK-II). The possible binding mode of TDZD-8 and GSK-3β was proposed based on the 3D crystalline structure of GSK-3β (Figure 1.7) (Martinez et al., 2005; Martinez et al., 2002). The superior potency and selectivity makes TDZD-8 ideal for studying potential anti-inflammatory effects in a mouse asthma model through inhibition of GSK-3β and regulation of NF-κB pathways.

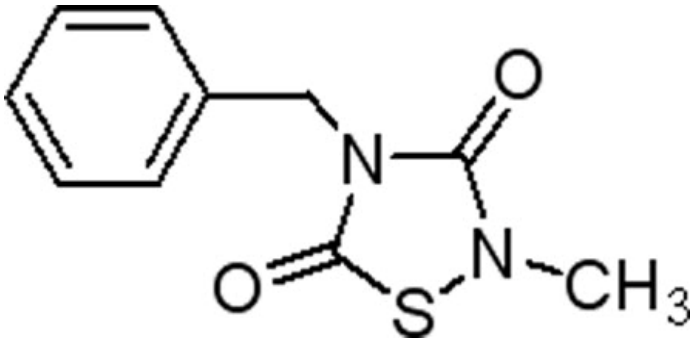


Figure 1.6 The Structure of TDZD-8 (Adapted from Martinez et al., 2002).

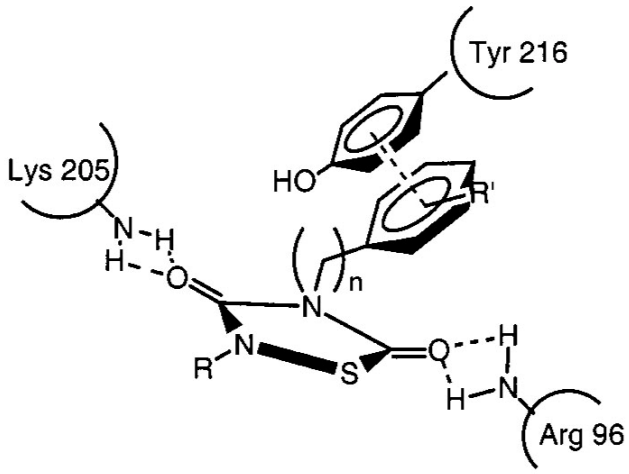


Figure 1.7 Proposed binding mode of TDZD-8 to GSK-3β (Adapted from Martinez et al., 2002).

1.3.2. Andrographolide

1.3.2.1. Introduction of andrographolide

Andrographis paniculata Nees (Family, Acanthaceae), also called the king of bitters due to its bitterness, is widely cultivated in southern Asia including China, India, Malaysia, Indonesia, Sri Lanka, etc (Figure 1.8) (Negi et al., 2007). This plant is known by various vernacular names such as Chuan Xin Lian in China, Kalmegh and Bhunimba in India, and Hempedubumi in Maylaysia. In addition, andrographis paniculata has been used as a herbal medicine for a long time in Asia and Scandinavia. The leaves and stems of this plant are used to extract the active phytochemicals. The active components of andrographis paniculata include andrographolide, neoandrographolide, andrographoside, and neoandrographoside (Figure 1.9). Although each component possesses distinct features in terms of pharmacological activity, andrographolide is the most active and documented component in this plant. Andrographolide is present in all parts of andrographis paniculata, especially in the leaves (around 2%) (Negi et al., 2007). Chemically, andrographolide is named as 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8α-dimethyl-2-methylene-1- napthalenyl]ethylidene]dihydro-4-hydroxy-2(3H)-furanone, and its structure and stereochemistry have been intensively studied (Chakravarti and Chakravarti, 1951; Pramanick et al., 2006). Andrographolide is also called diterpene lactone, a chemical name that describes its ring-like structure.

Figure 1.8 Andrographis paniculata.

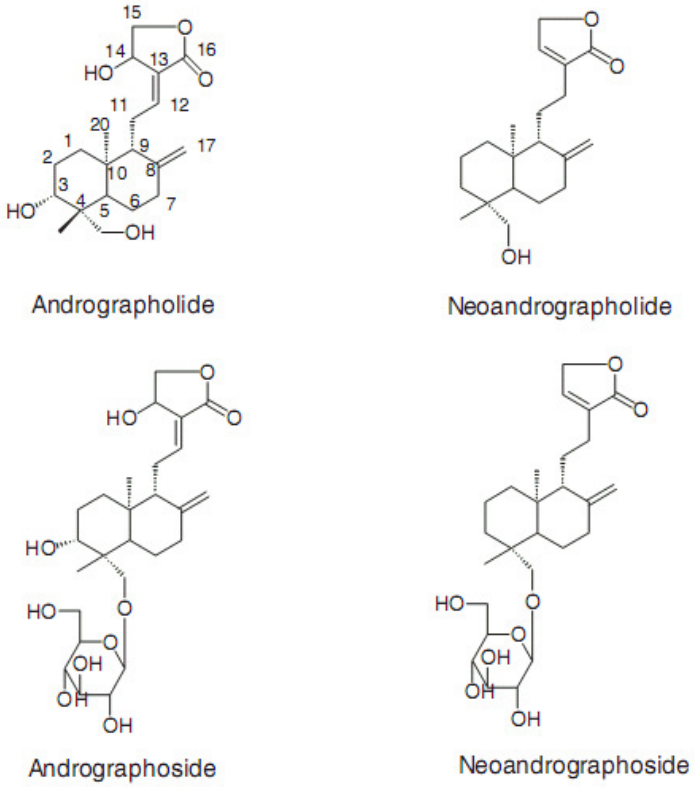


Figure 1.9 Major active components of andrographis paniculata.

1.3.2.2. Andrographolide and the NF-κB pathway

Andrographolide has shown a wide range of therapeutic effects such as anti-viral (Calabrese et al., 2000), anti-thrombotic (Thisoda et al., 2006; Zhao and Fang, 1991), anti-cancer (Jiang et al., 2007; Liang et al., 2008; Rajagopal et al., 2003; Sheeja and Kuttan, 2007a; Zhao et al., 2008), hepatoprotective (Jain et al., 2000; Singha et al., 2007; Visen et al., 1993), and modulation of immune responses (Sheeja and Kuttan, 2007b; Xu et al., 2007). More recently, it has been suggested that andrographolide also has anti-inflammatory activities. Andrographolide was thought to suppress the migration of inflammatory cells by reducing the expression of adhesion molecules (Wang et al., 2007). In addition, in RAW cells, upon stimulation by LPS and IFNγ, protein level and enzyme activity of inducible nitric oxide synthase (iNOS) were reduced following treatment with andrographolide (Chiou et al., 2000). Furthermore, andrographolide reduces LPS induced microglia damage by inhibiting proinflammatory cytokines such as iNOS and COX-2 (Wang et al., 2004) and suppresses the production of both TNF-α and IL-12 in LPS stimulated murine peritoneal macrophages (Qin et al., 2006).

Although the anti-inflammatory effects of andrographolide is well established, little is known about the exact mechanisms of this compound and various hypotheses have been proposed. One convincing observation of the anti-inflammatory activity of andrographolide is via the inhibition of NF-κB pathways reported by Xia and coworkers (2004). They showed that andrographolide could

bind to reduced cys^{62} of p50, inhibiting the formation of p50/p65 hererodimer and NF-κB transcriptional activity (Xia et al., 2004). Inhibitory effects of andrographolide on NF-κB activity is further supported by experiments on an arterial injury model (Wang et al., 2007) and neutrophilic cells (Hidalgo et al., 2005). Furthermore, andrographolide has been found to suppress the expression of various molecules such as iNOS, COX-2, E-selectin, VCAM-1, etc, which are all regulated by NF-κB based pathways (Kumar et al., 2004). Therefore, it is essential to find out if andrographolide would exert anti-inflammatory effects in a mouse asthma model and to explore underlying anti-inflammatory mechanisms.

1.4. The animal model of asthma

Experimental models of asthma have contributed greatly to the understanding of the pathogenesis of asthma and the development of new therapies for past decades (Zosky and Sly, 2007). Many species of animals are commonly used as models to investigate asthma, including mice, rats, guinea pigs, monkeys, ferrets, cats, dogs, pigs, and horses (Kurucz and Szelenyi, 2006). Generally, every species has its pros and cons, and species-specific differences include anatomical properties, and physiological and immunological responses (Zosky and Sly, 2007).

Among these asthmatic models, mouse models of asthma are, undoubtedly, the most popular choice for asthma studies (Zosky and Sly, 2007). The mouse model mimics many features of human asthma. After sensitization and challenge with antigens, mouse asthmatic models display a classical Th2 type immunologic

response with lung eosinophilia, high antigen-specific IgE and IgG, mucus hypersecretion, and AHR (Zosky and Sly, 2007). Furthermore, due to the availability of wild-type, mutant, knockout and transgenic mice, it is easy to investigate functions of a specific gene in the initiation and development of asthma in the mouse model. In addition, there are a wide variety of immunological reagents available for the study of mouse species. Finally, studies on mouse asthmatic models have highlighted the importance of Th2 cytokines such as IL-4, IL-5, and IL-13, in allergic responses. Therefore, we chose the mouse asthmatic model to investigate the anti-inflammatory effects of TDZD-8 and andrographolide in relation to NF-κB signaling pathways.

We used a typical sensitization and challenge protocol with female BALB/c mouse. BALB/c mouse is a high-responder mouse strain in which both airway inflammation and AHR are easily induced. The immune system shows a marked gender differences, for instance women are more susceptible to autoimmune and inflammatory diseases (Da Silva, 1999). Female mice displayed a higher serum IgE level and are more susceptible to airway inflammation than male mice (Corteling and Trifilieff, 2004; Hayashi et al., 2003; Melgert et al., 2005). We used OVA as the antigen for asthma model, because it has been produced via the standardized protocol by the industry and is easily manipulated to avoid the exposure to mouse immune system before sensitization. Aluminium hydroxide

was used as the adjuvant to promote the Th2 responses of the immune system in the mouse asthma model (Brewer et al., 1999).

However, no animal model could completely mimic asthma in humans and each model has its limitations and advantages (Epstein, 2004). For instance, the mouse model lacks the chronic inflammatory response to long-term challenge with antigens. Moreover, immunological responses in mouse asthmatic models are even being suppressed by long-term challenge following sensitization due to immune tolerance (Epstein, 2004). Therefore, we should keep these limitations in mind when we design studies and interpret results from animal models.

2. RATIONALE AND OBJECTIVES

Asthma is a chronic airway disease which affects around 300 million people of all ages and ethnic backgrounds (Masoli et al., 2004). The pathophysiology of asthma is multifactorial, involving an intricate network of molecular and cellular interactions. Besides, both genetic background and environmental factors also contribute to the initiation and development of asthma (Busse and Lemanske, 2001).

Current therapies for asthma mainly rely on β2 agonists and inhaled corticosteroids. While inhaled corticosteroids are at present the most effective anti-inflammatory agents for asthma, major concerns about the systemic effects of inhaled corticosteroids remain. Furthermore, up to 15% of patients who suffer from uncontrollable asthmatic symptoms do not respond well to corticosteroids (Amon et al., 2006). There is an urgent need to discover and develop novel and potent anti-inflammatory drugs for asthma.

Advances in the understanding of the pathophysiology of asthma have shed light on the development of novel therapies for asthma. The NF-κB family plays a key role in the initiation and development of asthma (Kumar et al., 2004). Therefore, the development of specific inhibitors targeting the NF-κB signaling pathway is promising for attenuating allergic responses. Nevertheless, because the NF-κB transcription factors are central to both normal biological functions and pathological conditions, direct inhibition of NF-κB per se may not be a safe approach. Rather, appropriate and specific inhibition of signaling molecules that

regulate NF-κB may be an effective anti-inflammatory strategy for asthma (Uwe, 2008).

GSK-3β has been known to regulate the phosphorylation of p65 in the NF-κB pathway and andrographolide has been shown to interfere with the activity of NF-κB pathway. The objectives of my thesis project were to examine the potential anti-inflammatory effects of a GSK-3β inhibitor, TDZD-8 (Martinez et al., 2002) and a herbal medicinal, andrographolide (Negi et al., 2007)) in a mouse asthma model and elucidate their mechanisms in the regulation of NF-κB pathway. In the future, the aims are to develop efficacious therapies that are easy to comply with or have minimal systemic side effects.

3. MATERIALS AND METHODS

3.1. Materials and reagents

Drugs and chemicals used in this PhD project are as follows: 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8α-dimethyl-2-methylene-1-napthalenyl]ethylidene]dihydro-4-hydroxy-2(3H)-furanone (andrographolide) , anti-β-actin monoclonal antibody, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), 10% neutral buffered formalin, acetyl-β-methylcholine chloride, aluminium hydroxide ($Al(OH)_3$), bovine serum albumin (BSA), chicken ovalbumin (OVA), dimethyl sulfoxide (DMSO), eosin Y, Harris hematoxylin solution, hematoxylin solution Gill no. 3, heparin, periodic acid solution, Schiff's reagent, and Tween 20 (Sigma-Aldrich, St Louis, MO, USA); RNAlater (Applied Biosystems, Foster City, CA, USA); alkaline phosphatase (AP) conjugate substrate kit, agarose, blotting paper, polyvinylidene difluoride (PVDF) membrane, tetramethylethylenediamine (TEMED), 3,3´,5,5´-tetramethylbenzidine (TMB) substrate kit, Triton X-100 (Bio-Rad Laboratories, Hercules, CA, USA) ammonium chloride (NH_4Cl) (BDH Laboratory Supplies, Poole, England); agarose, phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS), and tris-acetate-EDTA (TAE) (1st BASE, Singapore); diethylpyrocarbonate (DEPC)-treated water, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, penicillin, L-glutamine, streptomycin, random primer, Roswell Park Memorial Institute (RPMI) medium, Trizol, and trypan blue (Invitrogen, Carlsbad, CA, USA); anti-mouse IL-13 monoclonal antibody, biotinylated anti-mouse IL-13 antibody, recombinant murine

IL-13, anti-mouse eotaxin, biotinylated anti-mouse eotaxin, and recombinant murine eotaxin (R&D Systems, Minneapolis, MN, USA); avidin-horseradish peroxidase (HRP), biotinylated anti-mouse IgE, biotinylated anti-mouse IgG1, biotinylated anti-mouse IgG2a, cell strainer, fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody, FITC-conjugated anti-CD8 antibody, FITC-conjugated anti-B220 antibody, FITC-conjugated anti-NK1.1 antibody, OptEIA^{TM} mouse IFNγ set, OptEIA^{TM} mouse total IgE set, OptEIA^{TM} mouse IL-4 set, OptEIA^{TM} mouse IL-5 set, and phycoerythrin (PE)-conjugated anti-CD3 antibody (BD Biosciences Pharmingen, San Diego, CA, USA); bicinchonic acid (BCA) protein assay kit, calf serum, fetal bovine serum (FBS), infinity^{TM} alanine aminotransferase (ALT) liquid stable reagent, infinity^{TM} aspartate aminotransferase (AST) liquid stable reagent, Restore^{TM} PLUS western blot stripping buffer (Thermo Fisher Scientific Inc, Waltham, MA ,USA); sodium carbonate (Na_2CO_3) (Kanto Chemical, Tokoyo, Japan); absolute ethanol, isopropanol (Merck, Darmstadt, Germany); HistoClear, and Histomount (National Diagnostics, Atlanta, GA, USA); Non-fat milk powder (Nestle, Switzerland); avian myeloblastosis virus (AMV) reverse transcriptase, dNTP mix, polymerase chain reaction (PCR) master mix, and ribonuclease inhibitor (Promega, Madison, WI, USA); ethidium bromide (Research Organics, Cleveland, OH, USA); anti-IκB-α polyclonal antibody, anti-phospho-IκB-α ($Ser^{32/36}$) monoclonal antibody, anti-MEK1/2 polyclonal antibody, anti-phospho-MEK1/2 ($Ser^{217/221}$) polyclonal antibody, anti-IKKβ polyclonal antibody, anti-phospho-IKKβ (Ser^{180}) antibody, anti-p44/42 MAP kinase polyclonal antibody,

anti-phospho-p44/42 (Thr^{202}/Tyr^{221}) polyclonal antibody, anti-phospho-p65 (Ser^{536}) monoclonal antibody, and anti-p65 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA); enhanced chemiluminescent (ECL) western blotting detection reagents, and hyperfilm (GE Healthcare, Piscataway, NJ, USA); protease inhibitor cocktail (complete) (Roche Diagnostics, Indianapolis, IN, USA); HRP-conjugated anti-mouse Ig, HRP-conjugated anti-rabbit Ig, AP-conjugated anti-mouse Ig, and AP-conjugated anti-rabbit Ig (Dako, Glostrup, Denmark); TNF-α (BioSource, Camarillo, CA, USA); and nuclear extract kit, TransAM™ NF-κB p65 Kit (ActiveMotif, Carlsbad, CA, USA);

3.2. Asthma mouse model

All female BALB/c mice, 6-8 wk of age (Interfauna, East Yorkshire, UK) for this PhD project were ordered from center for animal resources and housed in plastic cages (maximum 4 mice/cage) in Animal Holding Unit in the National University of Singapore according to the spirit of Good Laboratory Practice. Female BALB/c mice are high responders in which both IgE response to OVA and airway response to mechacholine are easily induced (Corteling and Trifilieff, 2004). Briefly, animal rooms should be regulated by automatic timers to provide cycles with 12-14 hours of light and 10-12 hours of dark. The temperature in the animal room should range from 18° to 26°C with an average temperature of 22°C. Maintenance diets generally contain 4-5% fat and 14% protein. Generally a minimum of 3 days of acclimatization is required for mice to adapt their new surroundings.

Mice were sensitized with 20 μg OVA and 4 mg $Al(OH)_3$ in 0.1 ml saline by i.p on day 0 and day 14. For OVA Challenge, 0.15 g of OVA was dissolved in 15 ml of saline to be aerosolized by a DeVilbiss Ultra-Neb Large-Volume Ultrasonic Nebulizer (Sunrise Medical Respiratory Products, Somerset, PA) (Figure 3.1) . Mice were then challenged with 1% OVA aerosol for 30 min in a chamber on days 22, 23 and 24 (Figure 3.1). Average OVA aerosol particle size was less than 5 μm. Saline aerosol was used as a negative control. We changed the cage bedding three times a week. Under the strict control of cage environments and without additional bacterial and parasites` infections, the failure rate of OVA sensitization is less than 5 %. Animal experiments were performed according to the Institutional Guidelines for Animal Care and Use Committee of the National University of Singapore.

The GSK-3β inhibitor TDZD-8 (1, 3, and 10 mg/kg) or vehicle (10% DMSO) in 0.1ml was given by intravenous injection through the tail vein 1 hour before each OVA aerosol challenge on days 22, 23 and 24 (Dugo et al., 2005). Andrographolide (0.1, 0.5, and 1 mg/kg) or vehicle (2% DMSO) in 0.1ml was given by i.p. injection twice a day (one hour before and eleven hours after each OVA aerosol challenge) on days 22, 23 and 24 (Xie et al., 2004).

3.3. Measurement of AHR

Two parameters, lung resistance (RI) and dynamic compliance (Cdyn), were measured in an invasive system in a whole body plethysmography (Figure 3.2).

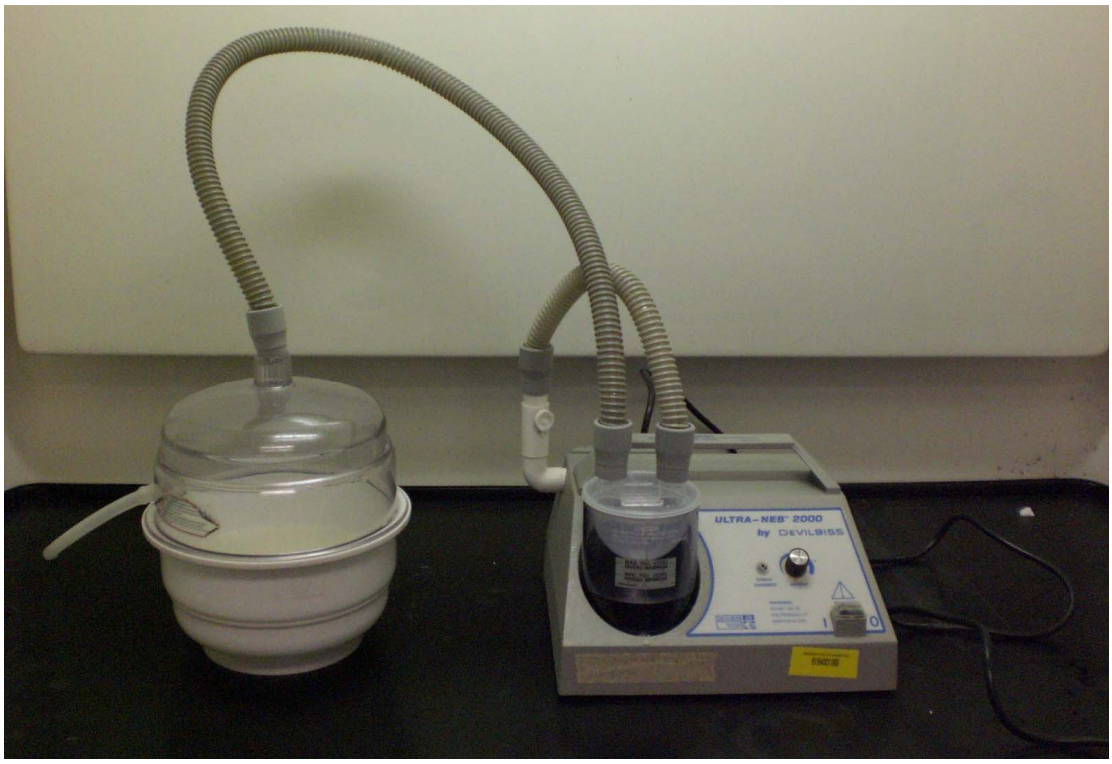


Figure 3.1 Aerosol delivery system. The ultrasonic nebulizer was employed to aerosolize 1% of OVA saline solution and pump the aerosol mist into an adjacent chamber where the aerosolized solution was inhaled into the airways of the mice. Up to 8 mice can be put into the chamber during OVA or saline challenge.

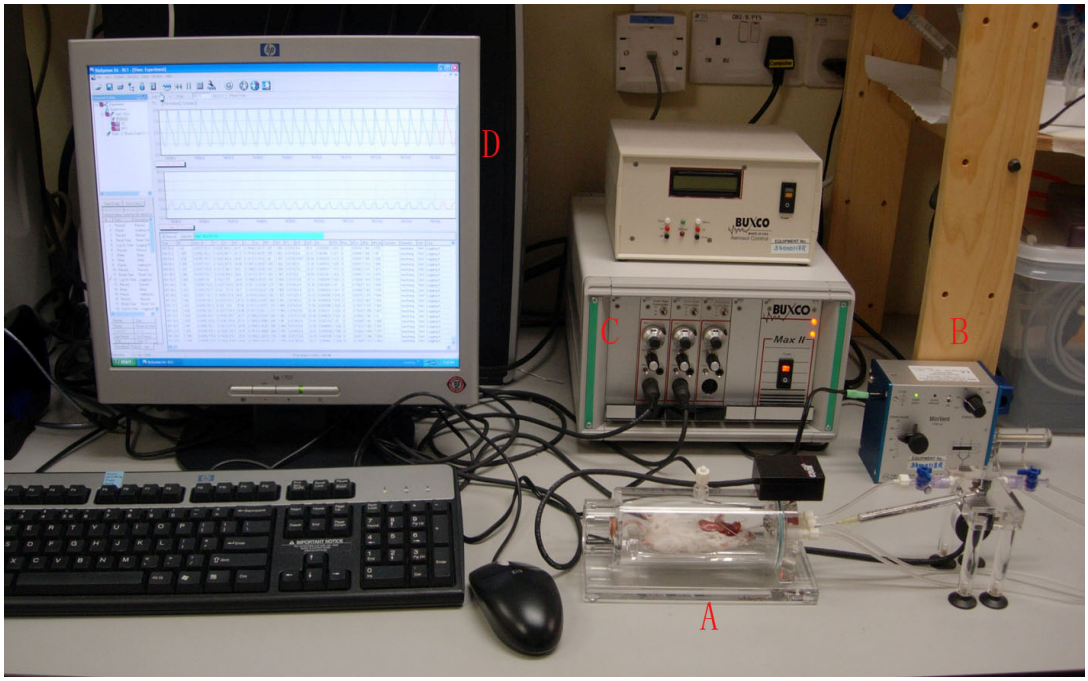


Figure 3.2 The Buxco system. (A) Chamber for anesthetized mice with ports to transducers. (B) Ventilator for supplying air to tracheostomized mice. (C) MAX Ⅱ amplifier for data acquisition. (D) BioSystem XA software with R/C analyzer to compute RI and Cdyn.

RI (ΔP/V) is defined as the pressure driving respiration divided by air flow. Cdyn (ΔV/ΔP) refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung.

AHR was measured 24 hours after the last saline or OVA challenge. Mice were anaesthetized by an i.p. injection of 200 μl of an anesthetic mixture (ketamine: medetomidine: H_2O = 3: 4: 33, Parnell, Alexandria NSW, Australia & Pfizer, Auckland, New Zealand). Then, a vertical cut of the skin above salivary glands was made and tissue under skin was pulled apart until the trachea was visible. The trachea was isolated by the forceps, a small cut was made by the scissors, and a tube (20G) was inserted into the trachea. Following that, the jugular vein was isolated by blunt dissection, cannulated by a blunt needle (27G), and connected to a microsyringe by a tube for intravenous methacholine administration. The mouse was placed in whole-body plethysmograph chamber (Buxco, Sharon, CT, USA) and ventilated mechanically by a ventilator (Hugo Sachs Elektronik, Harvard Apparatus, March-Hugstetten, Germany) via the tube which was already inserted into the trachea, at a tidal volume of 200 μl/breath and a breathing rate of 150/minute (Figure 3.2). Airflow changes in the sealed chamber and pressure changes in the airway were detected by the respective transducers, processed by a MAXⅡ amplifier, and analyzed by the Biosystem XA software (Buxco, Sharon, CT, USA). Methacholine was dissolved in PBS and administered through the jugular vein from a starting dose of 2.7 μg/kg to a final dose of 2000 μg/kg. The volume per methacholine dose was approximately 20 μl.

Threefold-increasing concentrations of methacholine were administered at 5 min intervals continuously. RI and Cdyn, in response to increasing concentrations of methacholine, were recorded and expressed as a percentage of the respective basal values in response to PBS.

3.4. Collection of bronchoalveolar lavage (BAL) fluid from mice

In separate groups of mice, BAL fluid was collected 24 hours after the last saline or OVA challenge as previously described (Duan et al., 2004). Mice were anaesthetized by an i.p. injection of 300 µl of an anesthetic mixture (ketamine: medetomidine: H_2O = 3: 4: 33, Parnell, Alexandria NSW, Australia & Pfizer, Auckland, New Zealand). Tracheotomy was carried out and a blunt needle (20G) was inserted into the trachea. Ice-cold PBS (0.5 ml × 3) was instilled into the lungs and a final volume of 1.1~1.3 ml of BAL fluid was retrieved from the lungs. According to preliminary experiments, BAL fluid cells retrieved from the same group were consistent if the final volume of BAL fluid was within the range from 1.1 to 1.3 ml.

3.5. Total and differential BAL fluid cell counts

The BAL fluid was centrifuged at 3000 rpm for 5 min at 4°C. Supernatant was collected and stored at -80°C until further experiments. The pellet was resuspended in 200 µl of 8.5 mg/ml NH_4Cl for 5 min at room temperature to remove red blood cells. The cell suspension was centrifuged at 3000 rpm for 5 min at 4°C and the supernatant was discarded. Afterwards, the cell pellet was resuspended in 200 µl of RPMI supplemented with 10 mg/ml BSA. A total number of viable cells was enumerated

using a haemocytometer (10 µl cell suspension: 10 µl 0.4% trypan blue) under a microscope (magnification ×200). Following the total cell count, aliquots (10^5 cells/150 µl) of the cell suspension were cytospined onto a slide in a Shandon Cytospin 3 (Thermo Electron Corporation, Pittsburgh, PA) at 600 rpm for 10 min at room temperature. The BAL fluid cells were stained with a modified Wright staining (Duan et al., 2004). Briefly, cytospin slides were fixed and stained with 800 µl of Liu A for 30 sec followed by 1600 µl of Liu B for 90 sec. Differential cell count was then performed on a minimum of 500 leukocytes (magnification ×1000). Four types of inflammatory cells, namely eosinophils, macrophages, neutrophils, and lymphocytes were identified and their respective percentage in the total inflammatory cells was enumerated, based on standard morphological criteria and staining (Figure 3.3). The absolute number of four types of inflammatory cells was calculated by their percentages and total inflammatory cell count. The epithelial cells were excluded in the differential cell count.

3.6. ELISA

3.6.1. Cytokines and chemokine levels in BAL fluid

Levels of IL-4, IL-5, IL-13, IFNγ and eotaxin in the supernatant of the BAL fluid were determined by ELISA (BD Biosciences Pharmingen, San Diego, CA, USA for IL-4 and IL-5; R&D Systems, Minneapolis, MN, USA for IL-13, eotaxin, and IFNγ) according to the manufacture`s instructions.

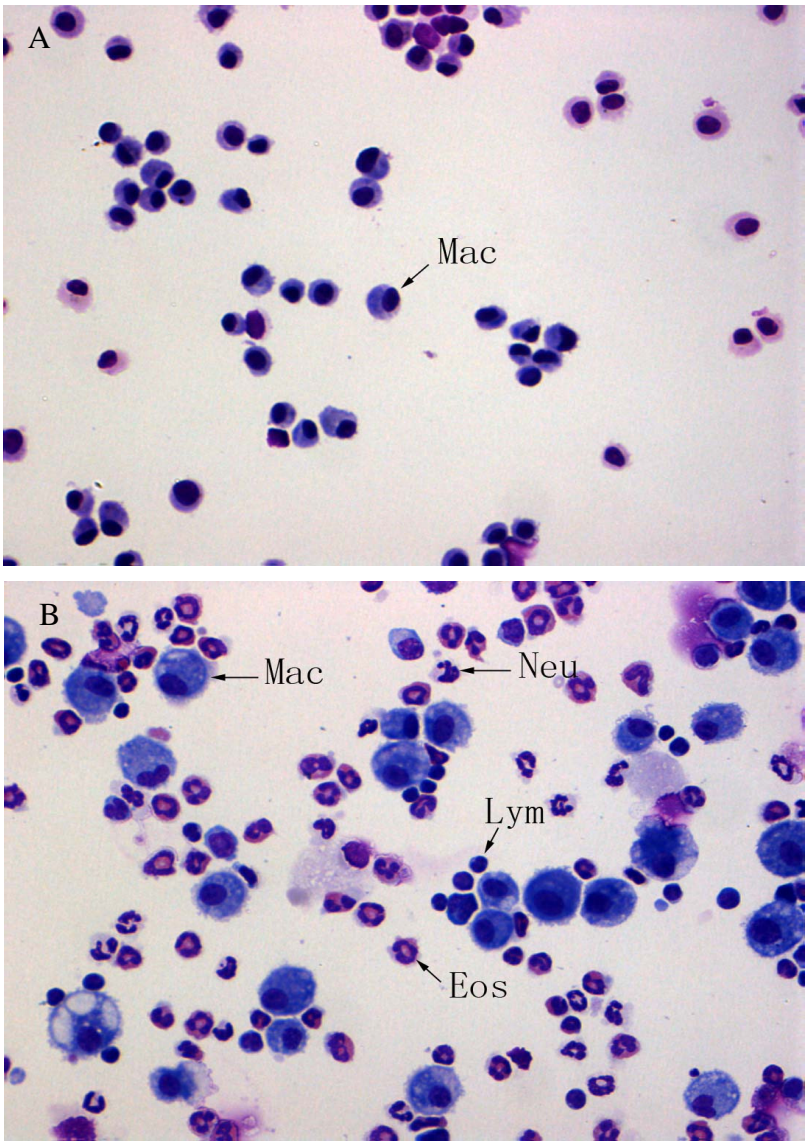


Figure 3.3 Type of cells found in BAL fluid of mice. (A) Control BAL fluid collect from the mouse sensitized with OVA and challenged with saline; (B) BAL fluid collected from the mouse sensitized with OVA and challenged with OVA. Mac, macrophages; Eos, Eosinophil; Lym, lymphocyte; Neu, neutrophil.

Briefly, 50 µl of diluted capture antibody (diluted to the appropriated concentration in relevant coating buffer) was coated to each well of an ELISA plate (NUNC, Denmark). The plate was sealed with parafilm and incubated overnight at 4°C. Next day, the coating buffer was aspirated and the plate was washed with wash buffer (PBS with 0.05% Tween-20). After washing, the plate was blocked with 300 µl PBS with 10% FBS for 2 hours. Following blocking, 50 µl of standards or BAL fluid samples were added into each well accordingly and incubated for 2 hours at room temperature. Next, after washing away the unbound samples and standards, the plate was incubated with biotinylated-detection antibody plus HRP for 1 hour (BD OptEIA^{TM} Kit) or with biotinylated-detection antibody for 1 hour followed by HRP for 45 min (R&D). Subsequently, after washing, 50 µl of a substrate solution was added to each well, and incubated for 30 min in dark, followed by addition of 50 µl stopping solution (1M H_2SO_4). Finally, the optical density of each well in the plate was read at 450 nm with λ correction at 570 nm within 30 min. The detection limits for the cytokines and chemokine used are as follows: 4 pg/ml for IL-4; 4 pg/ml for IL-5; 15.6 pg/ml for IL-13; 15.6 pg/ml for IFNγ; and 2 pg/ml for eotaxin.

3.6.2. Immunoglobulin levels in serum

Blood was collected from the mouse through cardiac puncture, and allowed to clot by leaving at room temperature for 3 hours. The clear supernatant was collected as the serum, and stored at -80°C until further experiments. Serum levels of total IgE, OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a were assayed by

ELISA. Briefly, ELISA plate was incubated at 4°C overnight with coating buffer containing either 20 μg/ml OVA (for OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a) or IgE capture antibody (for total IgE measurement). Next day, the plate was blocked with 10% FBS in 300 μl PBS for 2 hours at room temperature. Following blocking, total IgE standards or diluted serum samples were added into the respective wells and incubated for 2 hours. Next, the respective detection antibodies were added and the mixtures were incubated for 1 hour. After that, HRP-conjugated antibody was added and incubated for 45 min, followed by substrate solution for 30 min in the dark. Finally, the optical density of each well was read at 450 nm with λ correction at 570 nm within 30 min of adding stopping solution. The detection limit for total IgE is 2 ng/ml.

3.7. Histology

Lungs were isolated from the thoracic cavity 24 hours after the last OVA or saline challenge, fixed in 10% neutral buffered formalin solution for at least 48 hours, and processed in a tissue processor (Leica Microsystems, Wetzler, Germany). Briefly, lungs were dehydrated in a series of ethanol mixtures (70% → 80% → 90% → 100%, 30 min each and 2 hours for 100%), and immersed in xylene for 10.5 hours. Lungs were infiltrated with hot paraffin for 3 h and embedded in paraffin wax. The specimens were then cut into 5 μm sections using a microtome (Leica Microsystems, Wetzler, Germany) and fixed on slides.

For H&E staining, slides were deparaffinized with HistoClear for 10 min and rehydrated in a series of ethanol to water mixtures (100%→90%→70%→water and each for 2 min). The sections were then stained with Harris haematoxylin for 5 min, washed in the distilled water, and differentiated in 0.1% acid alcohol solution for 30 sec. The sections were washed with tap water for 5 min and counter stained with Eosin for 1 min. Afterwards, the sections were dehydrated in a series of ethanol solutions (70%→90%→100% and each for 30 sec) and immersed in HistoClear for 10 min. Evaluation of inflammation around peribronchial and perivascular areas was semi-quantitatively performed in a blind manner as previously described (Myou et al., 2003). A subjective scale (0 - 4) was assigned as follows: 0: no inflammatory cells; 1: occasional cuffing with few inflammatory cells; 2: most bronchi or vessels surrounded by a thin layer of inflammatory cells; 3: most bronchi or vessels surrounded by a thick layer (2 - 4 cells layer deep) of inflammatory cells; 4: most of bronchi or vessels surrounded by a thicker layer (more than 4 cells layer deep) of inflammatory cells.

Similarly, for periodic acid Schiff (PAS) staining, slides were deparaffinized with HistoClear for 10 min and rehydrated in a series of ethanol to water mixtures (100%→90%→70%→water and each for 2 min). Then, the sections were sequentially immersed in the periodic acid (5 min), water (5 min), and Schiff`s reagent (15 min). The sections were washed with tap water for 5 min and stained in Gill Haematoxylin for 90 sec. Next, the sections were dehydrated in a series of

ethanol mixtures (70%→90%→100% and each for 2 min) and cleared with HistoClear for 10 min. Evaluation of mucus production or goblet cell hyperplasia was semi-quantitatively performed in a blind manner as previously described (Grunig et al., 1998). According to the percentage of PAS-positive mucin-producing cells in the epthelium, scores (0 - 4) were assigned as follows: 0, no goblet cells; 1, < 25%; 2, 25–50%; 3, 50–75%; 4, > 75%.

For both H&E and PAS staining, bronchioles with the maximum internal diameter two times greater than the minimum internal diameter were not used for analysis. The scoring of inflammatory and goblet cells was performed in 2 - 4 preparations of each mouse and mean scores were calculated from 4 - 5 mice.

3.8. Western blotting

Lungs were isolated from the thoracic cavity 24 hours after the last OVA or saline challenge and stored in -80°C until study. Lung lobes were cut into small pieces using scissors and homogenized in ice-cold lysis buffer with a homogenizer (SilentCrusher M, Heidolph Elektro GmbH & Co, Kelheim, Genman). Lysates were then incubated on ice for 30 min before centrifugation (18,000g for 5 min); supernatants were collected, and protein concentrations were determined using a BCA protein assay kit. The same amount of protein (30 μg) mixed with sample buffer were separated by 10% SDS-PAGE in a Trans-Blot tank (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a PVDF membrane using a semi-try transblotter (ATTO Corp, Tokyo, Japan). The PVDF membrane was blocked with 5% non-fat milk in

Tween-20 Tris buffered saline (TTBS) for 2 hours and probed with anti-phospho-p65 (Ser^{536}), or anti-p65 antibodies for 2 hours. The PVDF was then washed with TTBS for 10 times (2 - 3 min each), and incubated in HRP-conjugated anti-rabbit or anti-mouse antibodies for one hour before being developed on hyperfilms using an ECL reagent. After washing several times in TTBS, ECL reagent was removed with western blot stripping buffer. Subsequently, the PVDF was blocked again, reprobed with anti-β-actin antibody, and developed using an AP substrate kit. Blots were quantitated using Gel-Pro v3.1 imaging software (Media Cybernetics, Silver Spring, MD, USA).

3.9. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Lungs were isolated from the thoracic cavity 24 hours after the last OVA or saline challenge and stored in RNAlater at -80°C. Before RNA isolation, lung tissues were removed from the RNAlater with sterile forceps, immersed in 1 ml Trizol, and homogenized on ice using the homogenizer. Then, RNA isolation was performed using Trizol according to the instructions from Invitrogen. After dissolving the isolated RNA in DEPC water, the spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific Inc, Waltham, MA, USA) was used to quantify the amount and purity of the RNA present in the sample. Both A260/A280 (DNA/protein) and A260/A230 (DNA/organic contaminants) ratio were recorded as an indicator of purity of RNA. An acceptable level of purity for RNA extracts should be around 1.9 to 2.1 for both A260/A280 and A260/A230 ratio. Then, cDNA was synthesized from 1 μg

of isolated RNA by a random primer and AMV reverse transcriptase using a multiwell thermal cycler (GeneAmp PCR system 2700, Applied Biosystems, Foster City, CA, USA). PCR amplifications were then performed on 1 µl of cDNA template in a 25 µl reaction volume containing 12.5 µl of 2× PCR master mix (50 units/ml TaqDNA polymerase, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM MgCl2), 9.5 µl nuclease-free water, 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM) using the multiwell thermal cycler. Primers used in the PCR reactions are listed in Table 3.1. PCR products underwent electrophoresis on 1.5% agarose gels stained with ethidium bromide (100 V, 30 min) and were visualized under an ultraviolet transilluminator. Furthermore, β-actin was used as a housekeeping gene to normalize variations between sample loadings.

3.10. Alanine and aspartate aminotransferase assay

Blood was collected from the mouse through cardiac puncture, and allowed to clot by leaving at room temperature for 3 hours. The clear supernatant was collected as the serum, and stored at -80°C until further experiments. Serum ALT and AST activities were measured using a kinetic spectrophotometric assay according to the manufacturer's instructions. Briefly, serum samples were kept at room temperature at least 3 days before the experiments. 10 µl sample and 100 µl ALT or AST reagents were mixed in 96 well plates and incubated for 30 seconds. Afterwards, absorbance value of each well was read at 340 nm with λ correction at 405 nm after exactly 1, 2, and 3 min, with constant temperature (37°C).

Table 3.1 Primer Sets for RT-PCR

Targets (Accession ID)		Primer Sequences	No. of Cycles	Length (bp)	Reference
TSLP (gi|10946697)	Forward Reverse	5'-CGACAGCATGGTTCTTCTCA-3' 5'-AAATGTTTTGTCGGGGAGTG-3'	28	265	Primer 3
ICAM-1 (gi|30172560)	Forward Reverse	5'-CATCGGGGTGGTGAAGTCTGT-3' 5'-TGTGGGGGAAGTGTGGTC-3'	25	624	(Lee et al., 2004)
VCAM-1 (gi|31981429)	Forward Reverse	5'-CAAGGGTGACCAGCTCATGAA-3' 5'-TGTGCAGCCACCTGAGATCC-3'	28	518	(Lee et al., 2004)
E-selectin (gi|118130193)	Forward Reverse	5'-AACGCCAGAACAACAATTCC-3' 5'- TGAATTGCCACCAGATGTGT-3'	30	227	Primer 3
AMCase (gi|37999744)	Forward Reverse	5'-TGGGTTCTGGGCCTACTATG-3' 5'-GCTTGACAATGCTGCTGGTA-3'	30	483	(Zhao et al., 2005)
Ym1 (gi|285015)	Forward Reverse	5'-CTGGAATTGGTGCCCCTACA-3' 5'-CAAGCATGGTGGTTTTACAGGA-3'	28	624	(Zhao et al., 2005)
Ym2 (gi|22123907)	Forward Reverse	5'-CAGAACCGTCAGACATTCATTA-3' 5'-ATGGTCCTTCCAGTAGGTAATA-3'	28	429	(Zhao et al., 2005)
YKL-40 (gi|142347793)	Forward Reverse	5'- GTACAAGCTGGTCTGCTACT-3' 5'- GTTGGAGGCAATCTCGGAAA-3'	29	277	(Mizoguchi , 2006)
Muc5ac (gi|114431223)	Forward Reverse	5'-GAGGGCCCAGTGAGCATCTCC-3' 5'-TGGGACAGCAGCAGTATTCAGT-3'	32	361	Primer 3
iNOS (gi| 146134510)	Forward Reverse	5'- GTCAACTGCAAGAGAACGGAGAC-3' 5'- GAGCTCCTCCAGACGGGTAGGCTTG -3'	33	456	Primer 3
IL-6 (gi|155369258)	Forward Reverse	5'- CAGGAGAAGATTCCAAAGAT -3' 5'- ACTGGTTCTGTGCCTGCAGC -3'	28	459	(Bao et al., 2007)
IL-8 (gi|28610153)	Forward Reverse	5'-ATGACTTCCAAGCTGGCCGTGGCT-3' 5'- TCTCAGCCCTCTTCAAAAACTTCTC-3'	27	292	(Bao et al., 2007)
RANTES (gi|22538813)	Forward Reverse	ATGAAGGTCTCCGCGGCACGCCT CTAGCTCATCTCCAAAGAGTTG	28	276	(Bao et al., 2007)
MCP-1 (gi|56119169)	Forward Reverse	5'-GATCTCAGTGCAGAGGCTCG-3' 5'-TGCTTGTCCAGGTGGTCCAT-3'	29	153	(Bao et al., 2007)
β-actin (gi|6671508)	Forward Reverse	5'-TCATGAAGTGTGACGTTGACATCCGT-3' 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'	22	285	Promega, USA

Activities of AST or ALT were calculated by the change in absorbance per min from the linear portion of the reaction curve (Δabsorbance/min*factor).

3.11. NF-κB transcription factor assay

Lungs were collected 24 hours after the last OVA or saline challenge and stored at -80°C until further study. Lungs were homogenized on ice using the homogenizer and nuclear fractions were isolated with an Active Motif nuclear extract kit according to the instructions from the manufacturer.Protein concentrations of the nuclear extracts were determined by the BCA protein assay. NF-κB DNA-binding activity was analyzed using the TransAM^{TM} NF-κB p65 transcription factor assay kit following the manufacturer's protocol. Briefly, this kit contains a 96-well plate with immobilized oligonucleotide containing the NF-κB consensus binding sequence (5'-GGGACTTTCC-3'). Nuclear extracts (5 µg) were incubated in the wells for 1 hour and anti-p65 primary antibodies were then added into the wells following washing. A HRP-conjugated secondary antibody was subsequently added to detect the bound primary antibody. Developing solution and stoping solution were used to develop a colorimetric reaction. The absorbance was measured at 450 nm with a reference wavelength of 655 nm by a microplate reader (Tecan group Ltd. Mannedorf, Switzerland). In addition, the wild-type or mutated NF-κB consensus oligonucleotide was added into the reactions as the competitive or mutated competitive control.

3.12. Cell cultures

3.12.1. Lymphocyte recall experiments

Bronchial lymph nodes (3 - 4 nodes per mouse) were isolated from the lungs aseptically and passed through cell strainers to prepare a single-cell suspension. Cells were cultured at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomcin, 25 mM HEPES buffer, and 10% heat-inactivated FCS. Cells were cultured in medium only, or with 200 μg/ml OVA. Con-A (10 μg/ml) was treated as a positive control. After 72 hours' incubation, the supernatants from triplicate cultures were collected at -80°C until analysis of cytokine concentrations by ELISA.

3.12.2. Normal human bronchial epithelial cells

Normal human bronchial epithelial cells (Cambrex BioScience, Walkersville, MD, USA) were grown in bronchial epithelial bulletkit medium (Cambrex BioScience, Walkersville, MD, USA) supplemented with bovine pituitary extract (2 ml), hydrocortisone (0.5 ml), recombinant human EGF (0.5 ml), epinephrine (0.5 ml), transferring (0.5 ml), insulin (0.5 ml), retinoic acid (0.5 ml), triiodothyronine (0.5 ml), gentamycin sulfate (50 μg/ml), and amphotericin B (50 ng/ml) in 5% CO2 incubator at 37°C, and subcultured at 80% to 85% confluency. One million cells were seeded onto the 100 mm cell culture plate 24 hours before the experiments. Cells were pretreated with 10 μM TDZD-8 or vehicle (0.05% DMSO) 1 hour before stimulation with 10 ng/ml TNF-α. Total protein and mRNA were extracted from cells 24 hours later.

3.12.3. A549 cells

A549 cells (human lung epithelial cell line, CCL - 185, American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI with 10% FBS in 5% CO2 incubator at 37°C, and subcultured at 80% to 85% confluency. One million of cells were seeded onto the 100 mm cell culture plate 24 hours before the experiments. Cells were pretreated with 30 μM andrographolide or vehicle (0.1% DMSO) for 4 hours before stimulation with 10 ng/ml TNF-α. The incubation time and andrographolide dose was determined by preliminary optimizing experiments. Total protein or nuclear protein was extracted at different time points and stored at -80°C until further experiments.

3.13. Statistical analysis

All data are presented as means ± SEM. One-way ANOVA followed by Dunnett`s test was used to determine significant difference between different groups using SPSS15 for windows (SPSS Inc, Chicago, IL, USA). The critical level for significance was set at $P < 0.05$.

4. ANTI-INFLAMMATORY EFFECTS OF A GLYCOGEN SYNTHASE KINASE-3B INHIBITOR, TDZD-8, IN A MOUSE ASTHMA MODEL

4.1. Results

4.1.1. Effects of TDZD-8 on OVA-induced eosinophil recruitment in BAL fluid

BAL fluid was collected 24 h after the last OVA or saline aerosol challenge, and total and differential cell counts were performed. We excluded the epithelial cells when we did the differential cell counts. In saline control mice, almost all BAL fluid leukocytes are macrophages. OVA inhalation significantly ($P < 0.05$) increased total cell, eosinophil and lymphocyte counts as compared with saline control (Figure 4.1). In contrast, macrophage and neutrophil counts were not significantly affected (Figure 4.1). TDZD-8 (1, 3 and 10 mg/kg) substantially reduced the total cell, eosinophil, and lymphocyte counts in BAL fluid in a dose-dependent manner as compared with the DMSO vehicle control (Figure 4.2). These results clearly suggest a possible role of GSK-3β inhibitor in eosinophil and lymphocyte recruitment during airway allergic inflammation. Flow cytometric analysis revealed that peripheral blood leukocytes from saline-challenged, OVA-challenged, vehicle control, and TDZD-8-treated mice had similar percentages of CD3+, CD4+, CD8+ T cells, B cells (B220), and NK cells (NK1.1) (Figure 4.3). Hence, inhibition of eosinophil and lymphocyte pulmonary recruitment is unlikely due to any potential non-specific cytotoxic effects of TDZD-8.

4.1.2. Effects of TDZD-8 on OVA-induced pulmonary cell infiltration and mucus production

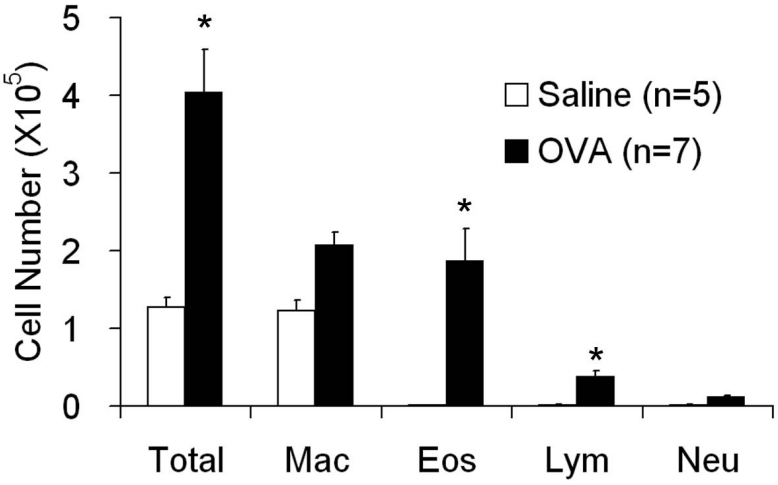


Figure 4.1 Differential cell counts in BAL fluid. Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 hours after the last saline aerosol or OVA aerosol challenge. Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Number of mice tested in each treatment group is shown as n value in parentheses. *Significant difference from saline control, $P < 0.05$.

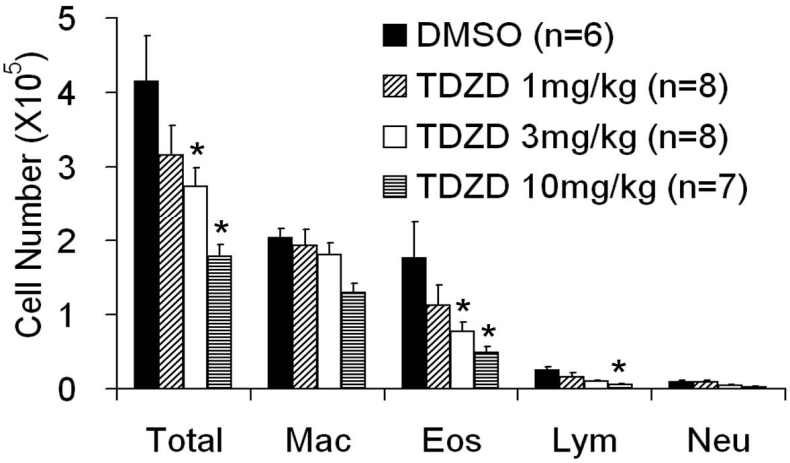


Figure 4.2 Effects of TDZD-8 on BAL fluid cell infiltration. TDZD-8 reduced OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 hours after the last OVA aerosol challenge. Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Number of mice tested in each treatment group is shown as n value in parentheses. *Significant difference from DMSO control, $P < 0.05$.

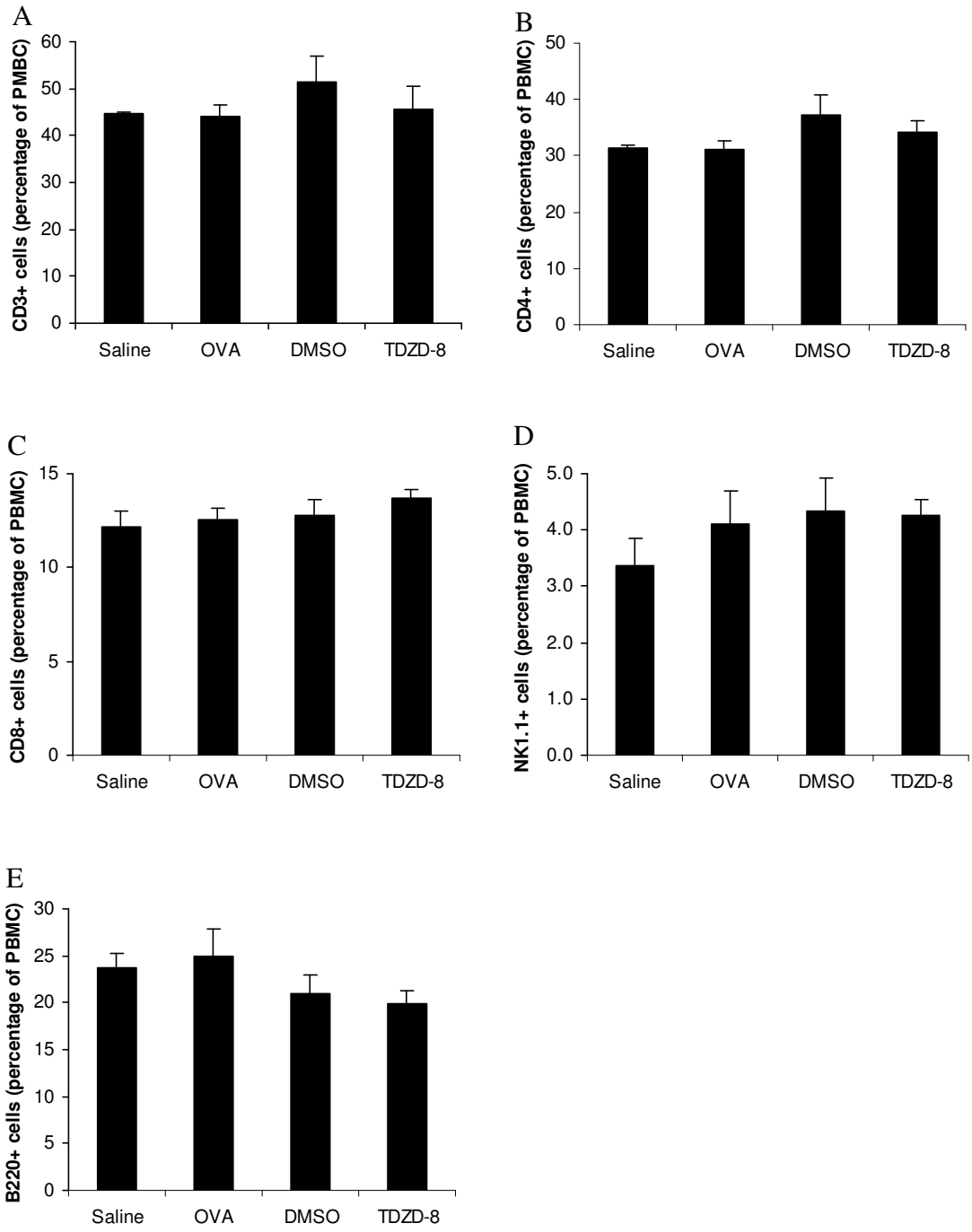


Figure 4.3 Effects of TDZD-8 on peripheral blood mononuclear cell (PBMC). Plasma was collected 24 hours after the last OVA or saline aerosol challenge. Expressions of (A) CD3, (B) CD4, (C) CD8, (D) NK1.1 and (E) B220 on PBMC were analyzed using FACS (n = 3 mice per group). The dose of TDZD-8 was 10 mg/kg. Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

Lung tissue was collected 24 h after the last OVA challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared to saline challenge (Figure 4.4 A and B). The majority of the infiltrated inflammatory cells were eosinophils.TDZD-8 (10 mg/kg) markedly attenuated the eosinophil-rich leukocyte infiltration as compared to DMSO control (Figure 4.4 C, D and I). On the other hand, OVA-challenged mice, but not saline-challenged mice, developed marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung (Figure 4.4 E and F). The OVA-induced mucus secretion was significantly attenuated by TDZD-8 (10 mg/kg) as compared to the DMSO control (Figure 4.4 G, H and J).

4.1.3. Effects of TDZD-8 on cytokine levels in BAL fluid

To determine the cytokine levels in the lung, IL-4, IL-5, IL-13 and eotaxin amounts in BAL fluid were measured using ELISA. As shown in Figure 4.5, OVA inhalation in sensitized mice substantially induced IL-4, IL-5, and IL-13 release into BAL fluid as compared with saline control. OVA moderately increased BAL fluid eotaxin level. TDZD-8 significantly ($P < 0.05$) decreased IL-5, IL-13 and, to a lesser extent, eotaxin levels in BAL fluid as compared with DMSO control (Figure 4.5 and Figure 4.6). In contrast, TDZD-8 had no effect on IL-4 levels in BAL fluid (Figure 4.5 A).

4.1.4. Effects of TDZD-8 on serum IgE levels

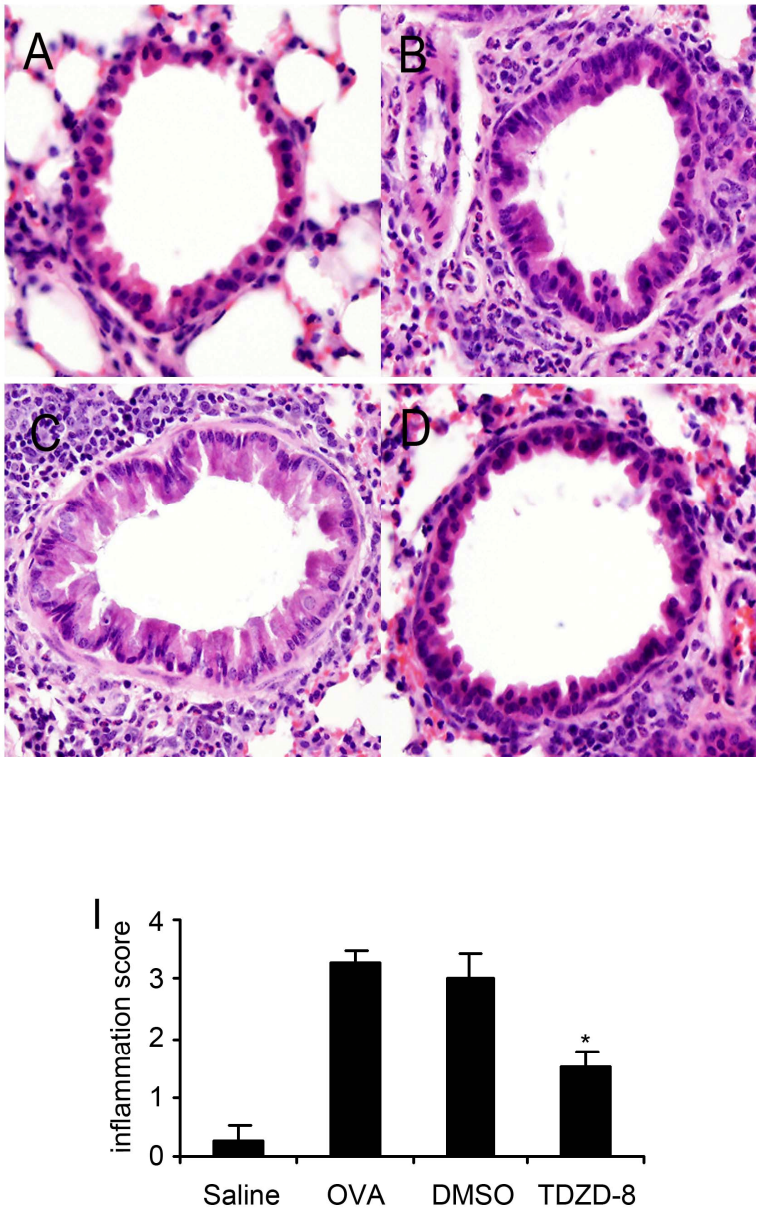


Figure 4.4 A-D, I Effects of TDZD-8 on lung tissue inflammatory cell infiltration. Histologic examination of lung tissue eosinophilia (A, B, C, and D, magnification ×400) 24 hours after the last challenge of saline aerosol (A), OVA aerosol (B), OVA aerosol plus DMSO (C), or OVA aerosol plus 10 mg/kg TDZD-8 (D). Quantitative analyses of inflammatory cell infiltration in lung sections were performed as described in chapter 3. Scoring of inflammatory cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $P < 0.05$.

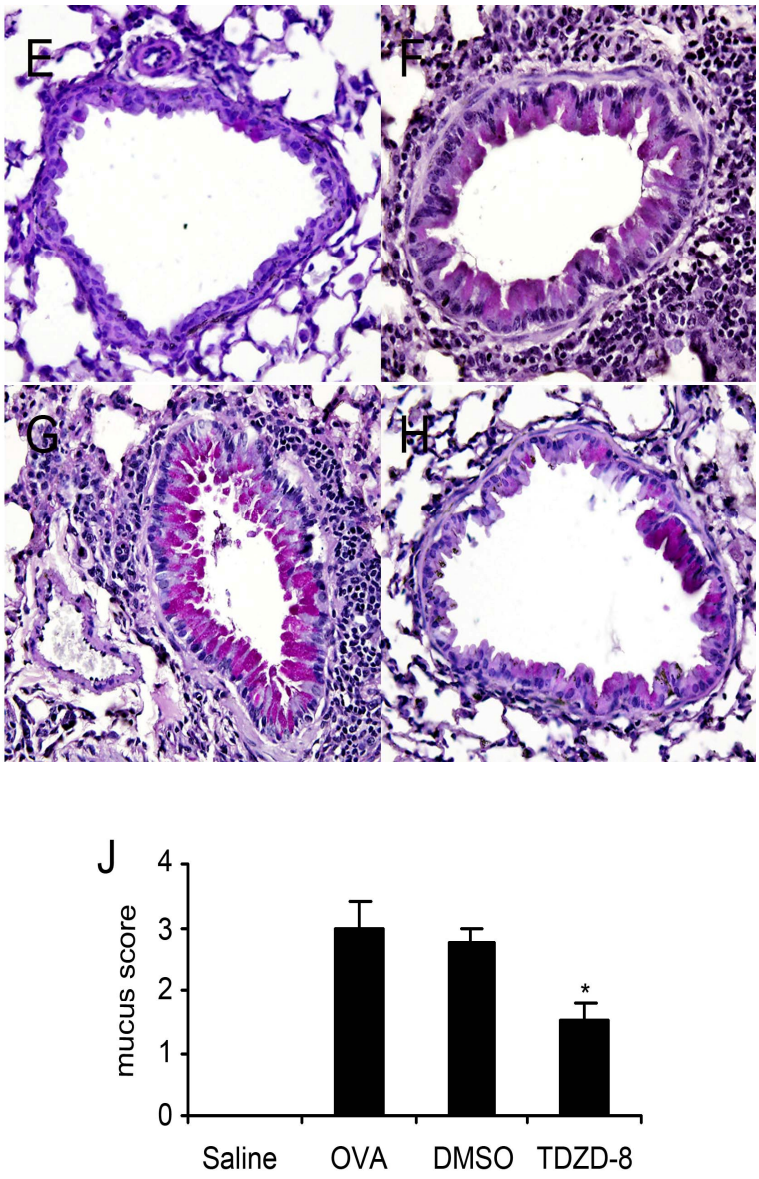


Figure 4.4 E-H, J Effects of TDZD-8 on mucus production. Histologic examination of mucus secretion (E, F, G, and H, magnification ×400) 24 hours after the last challenge of saline aerosol (E), OVA aerosol (F), OVA aerosol plus DMSO (G), or OVA aerosol plus 10 mg/kg TDZD-8 (H). Quantitative analyses of mucus production (J) in lung sections were performed as described in chapter 3. Scoring of goblet cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $P < 0.05$.

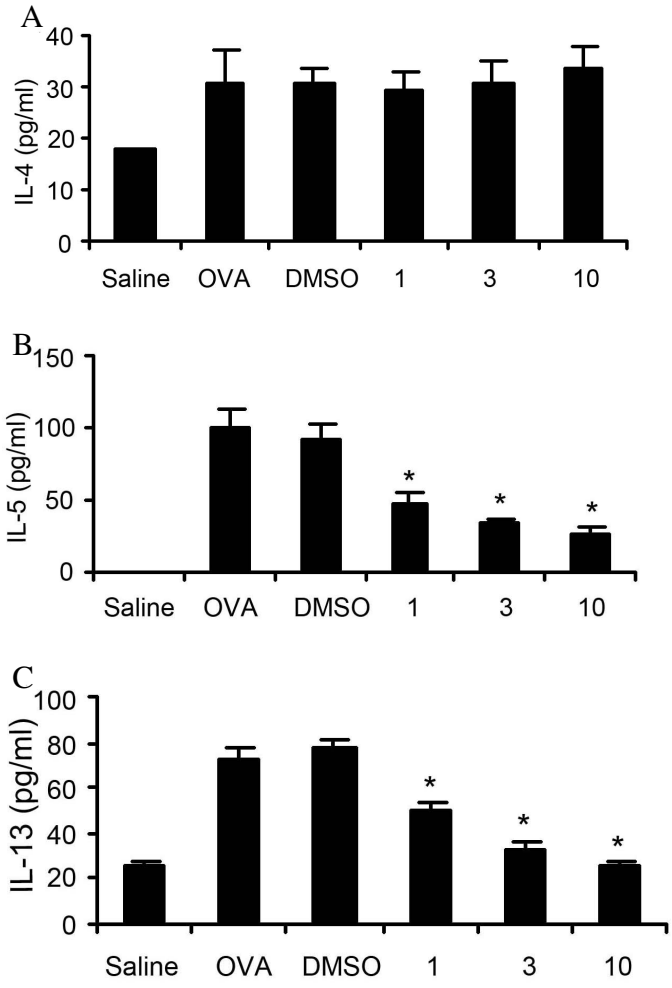


Figure 4.5 Effects of TDZD-8 on Th2 cytokines levels in BAL fluid. BAL fluids were collected 24 hours after the last OVA aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 1, 3, and 10 groups indicate asthmatic mice which were treated with three different doses of TDZD-8 (1 = 1 mg/kg, 3 = 3 mg/kg, and 10 = 10 mg/kg). Levels of (A) IL-4, (B) IL-5, and (C) IL-13 were analyzed using ELISA (n = 5 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

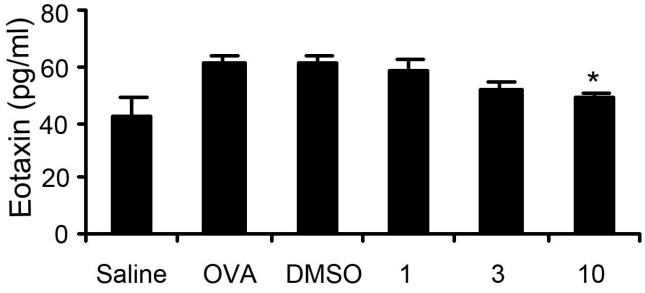


Figure 4.6 Effects of TDZD-8 on eotaxin level in BAL fluid. BAL fluids were collected 24 hours after the last OVA aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 1, 3, and 10 groups indicate asthmatic mice which were treated with three different doses of TDZD-8 (1 = 1 mg/kg, 3 = 3 mg/kg, and 10 = 10 mg/kg). The levels of eotaxin was analyzed using ELISA (n = 5 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

In order to evaluate whether TDZD-8 could modify an ongoing OVA-specific Th2 response in vivo, levels of total IgE and OVA-specific IgE were determined using ELISA. Substantial elevation in total IgE and OVA-specific IgE were observed in serum from OVA sensitized and challenged mice as compared to saline-challenged mice (Figure 4.7). TDZD-8 significantly ($P < 0.05$) lowered OVA-specific IgE levels at all three doses tested (Figure 4.7 A), but had no effect on the levels of total IgE (Figure 4.7 B), indicating an OVA-specific inhibition at the antigen presentation level or at the T cell- B cell interaction level Th2 response by the GSK-3β inhibitor.

4.1.5. Effects of TDZD-8 on lung mRNA expression of inflammatory markers

OVA aerosol challenge markedly up-regulated the mRNA levels of adhesion molecules ICAM-1 and VCAM-1 which are pivotal for pulmonary inflammatory cell recruitment such as eosinophils and lymphocytes (Jia et al., 1999); Muc5ac which is essential for mucin production (Zuhdi Alimam et al., 2000); and of the chitinase family members (acidic mammalian chitinase [AMCase], Ym1 and Ym2) which have recently been shown to play critical roles in airway inflammation and remodeling (Webb et al., 2001; Zhao et al., 2005; Zhu et al., 2004). Pre-treatment with TDZD-8 (10 mg/kg) moderately decreased OVA-induced ICAM-1, VCAM-1, and AMCase mRNA expression but dramatically suppressed Muc5ac, Ym1, and Ym2 in the allergic airway (Figure 4.8).

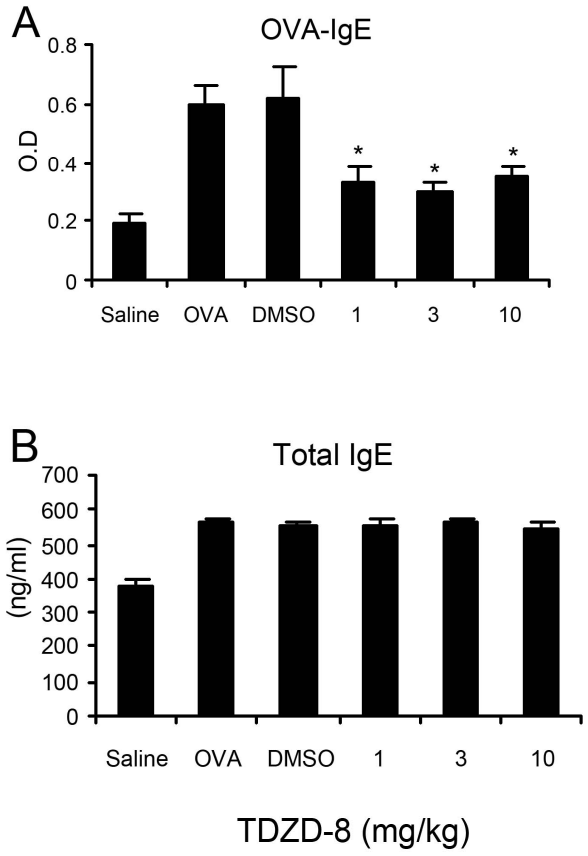


Figure 4.7 Effects of TDZD-8 on serum IgE production. Mouse serum was collected 24 hours after the last OVA aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 1, 3, and 10 groups indicate asthmatic mice which were treated with three different doses of TDZD-8 (1 = 1 mg/kg, 3 = 3 mg/kg, and 10 = 10 mg/kg). The levels of (A) OVA specific IgE and (B) total IgE were analyzed using ELISA (n = 6 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

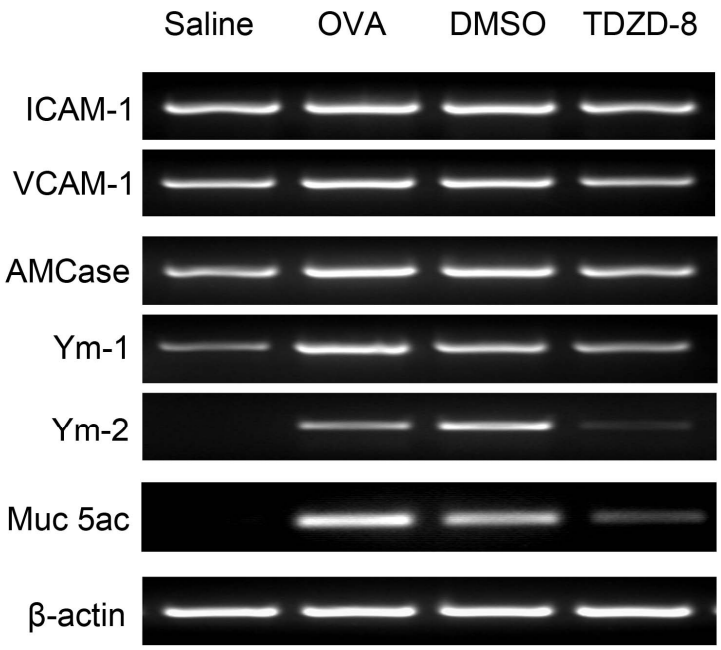


Figure 4.8 Effects of TDZD-8 on pulmonary mRNA expression of inflammatory markers. Lung tissues were collected 24 hours after the last OVA aerosol challenge. Total mRNA was extracted using TriZol reagent and the PCR products were separated in a 2% agarose gel visualized under ultraviolet light. β-actin was used as an internal control. The experiments were repeated for three times (n = 3 mice per group) with similar pattern of results.

4.1.6. Effects of TDZD-8 on OVA-induced AHR in mice

To investigate the effect of GSK-3β inhibition on AHR in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice with and without pretreatment of TDZD-8. As compared to saline-challenged group, OVA-challenged mice developed AHR which was typically reflected by high RI (Figure 4.9) and low Cdyn (Figure 4.10). TDZD-8 (10 mg/kg) dramatically suppressed AHR in OVA-challenged mice in response to methacholine, suggesting that Th2 immune responses and airway inflammation in vivo was modified (Figure 4.9 and Figure 4.10). DMSO did not show any inhibitory effects on AHR in OVA-challenged mice (Figure 4.9 and Figure 4.10).

4.1.7. Immunoblot analysis of lung NF-κB p65

To verify that the inhibitory effects of TDZD-8 on the mouse asthma model were mediated by NF-κB p65 inhibition in vivo, we examined the effects of TDZD-8 on OVA-induced NF-κB subunit p65 phosphorylation (Ser^{536}) in lungs isolated from mice treated with DMSO or 10 mg/kg TDZD-8. At 24 hours after the last OVA aerosol challenge, the extent of p65 (Ser^{536}) phosphorylation was similar to the one in saline-challenged control mice (data not shown). This likely implies that the phosphorylation event has already returned to basal level by 24 hours. Therefore, we tested the p65 (Ser^{536}) phosphorylation 2 hours after the last OVA challenge.

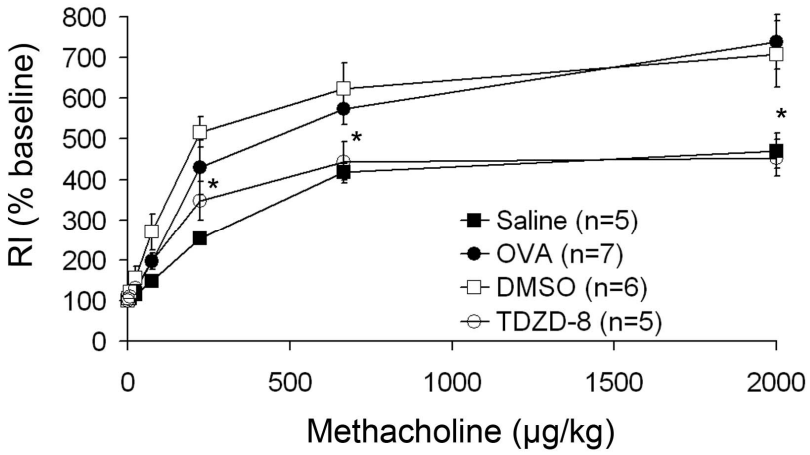


Figure 4.9 Effects of TDZD-8 on airway resistance. Airway responsiveness of mechanically ventilated mice in response to intravenous methacholine was measured 24 hours after the last saline aerosol or OVA aerosol with pretreatment of either DMSO or 10 mg/kg TDZD-8. AHR is expressed as percentage change from the baseline level of lung resistance (RI). Number of mice tested in each treatment group is shown as n value in parentheses. *Significant difference from DMSO control, $P < 0.05$.

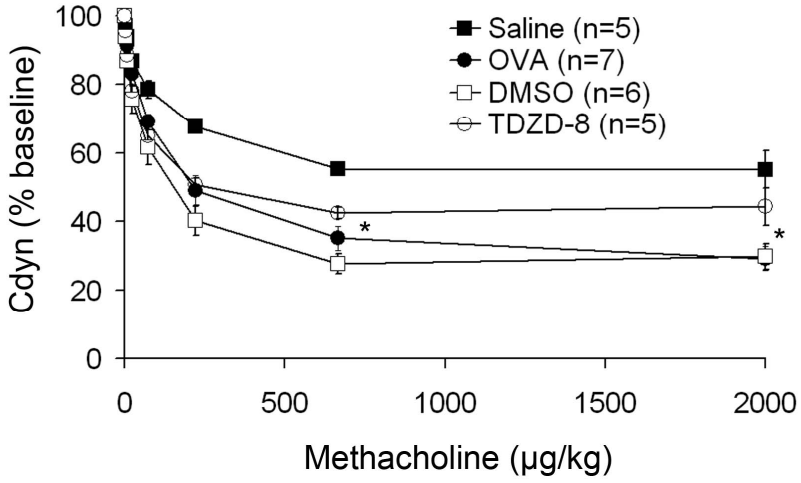


Figure 4.10 Effects of TDZD-8 on airway dynamic compliance. Airway responsiveness of mechanically ventilated mice in response to intravenous methacholine was measured 24 hours after the last saline aerosol or OVA aerosol with pretreatment of either DMSO or 10 mg/kg TDZD-8. AHR is expressed as percentage change from the baseline level of lung dynamic compliance (Cdyn). Number of mice tested in each treatment group is shown as n value in parentheses. *Significant difference from DMSO control, $P < 0.05$.

At this point, we were able to capture a significant ($P < 0.05$, when compared with saline- challenged mice) increase in p65 phosphorylation in the lung lysates from OVA-challenged mice, and this increase in p65 phosphorylation was significantly (when compared with DMSO vehicle control) inhibited by TDZD-8 (Figure 4.11). Total levels of NF-κB subunit p65 in lungs were not altered at all by GSK-3β inhibition. Equal loading of proteins was confirmed using β-actin as an internal control.

4.1.8 Effects of TDZD-8 on TNF-α stimulated human bronchial epithelial cells

To further characterize the anti-inflammatory effects of GSK-3β inhibition in a relevant airway cell type, we studied the effects of TDZD-8 on TNF-α (10 ng/ml) induced phosphorylation of NF-κB subunit p65 and cytokine mRNA expression in normal human bronchial epithelial cells. TNF-α has been implied to play a critical role in asthma (Erin et al., 2006; Hart et al., 1998) and is a potent stimulator of human airway epithelial cells (Newton et al., 2007). TNF-α drastically increased the p65 (Ser^{536}) phosphorylation, but did not affect the level of total p65 protein in normal human bronchial epithelial cells (Figure 4.12). TNF-α also up-regulated the mRNA level of proinflammatory cytokines, such as IL-6, IL-8, MCP-1, and RANTES, in cells (Figure 4.13). TDZD-8 (10 μM) markedly blocked TNF-α induced p65 (Ser^{536}) phosphorylation as well as the up-regulation of IL-6, IL-8, MCP-1, and RANTES in normal human bronchial epithelial cells (Figure 4.12 and Figure 4.13).

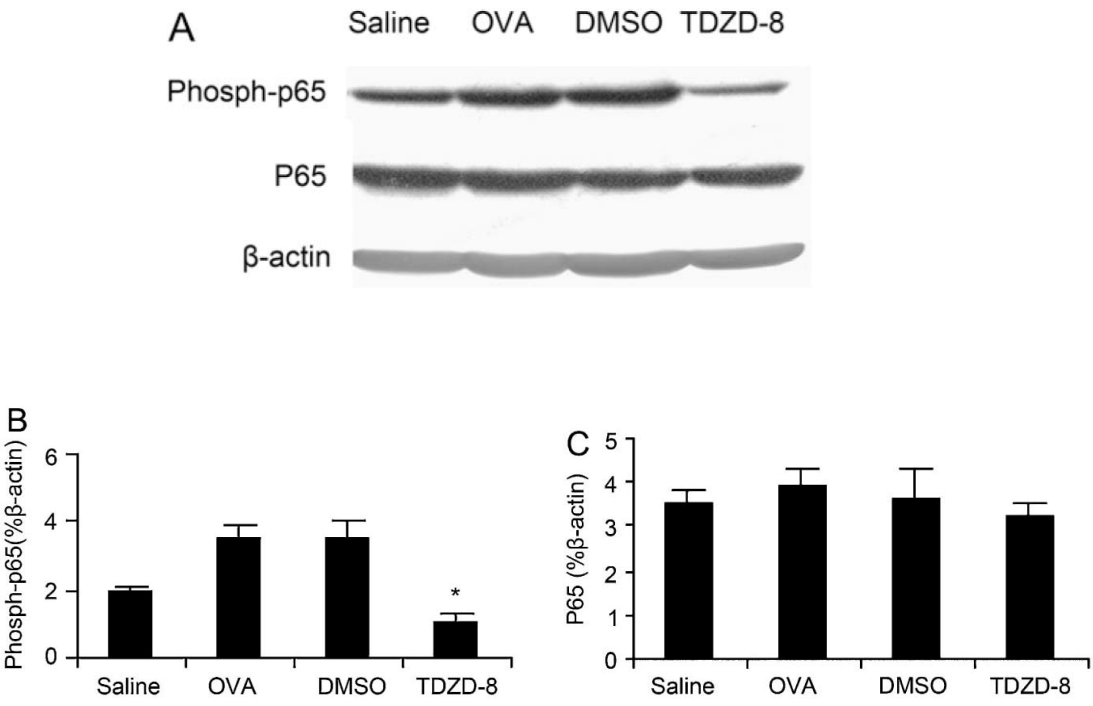


Figure 4.11 Effects of TDZD-8 on NF-κB subunit p65 phosphorylation in lung tissue. Western blot analysis of p65 phosphorylation at Ser^{536} in lung fragments isolated from mice 2 hours after the last saline aerosol or OVA aerosol challenge pretreated with either DMSO or 10 mg/kg TDZD-8. Proteins (10 μg per lane) were separated by SDS-PAGE and probed with anti-p65 or anti–phospho-p65 antibody. The blot was developed by enhanced chemiluminescence reagent and quantitated using Gel-Pro imaging software. β-actin was used as an internal control. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $P < 0.05$.

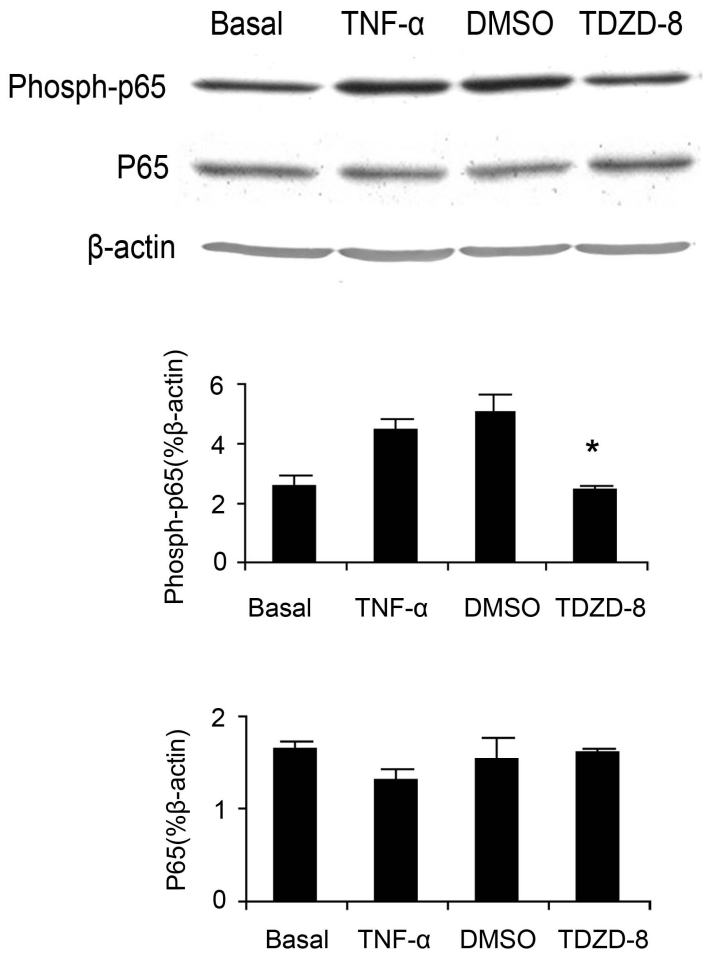


Figure 4.12 Effects of TDZD-8 on TNF-α-induced phosphorylation of p65 in normal human bronchial epithelial cells. Primary epithelial cells were stimulated with 10 ng/ml TNF-α for 24 hours, with or without pretreatment of 10 μM TDZD-8. Western blot analysis of p65 phosphorylation at Ser^{536} in human bronchial epithelial cells. Proteins (10 μg per lane) were separated by SDS-PAGE and probed with anti-p65 or anti–phospho-p65 antibody. The blot was developed by ECL reagent and quantitated using Gel-Pro imaging software. β-actin was used as an internal control. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $P < 0.05$.

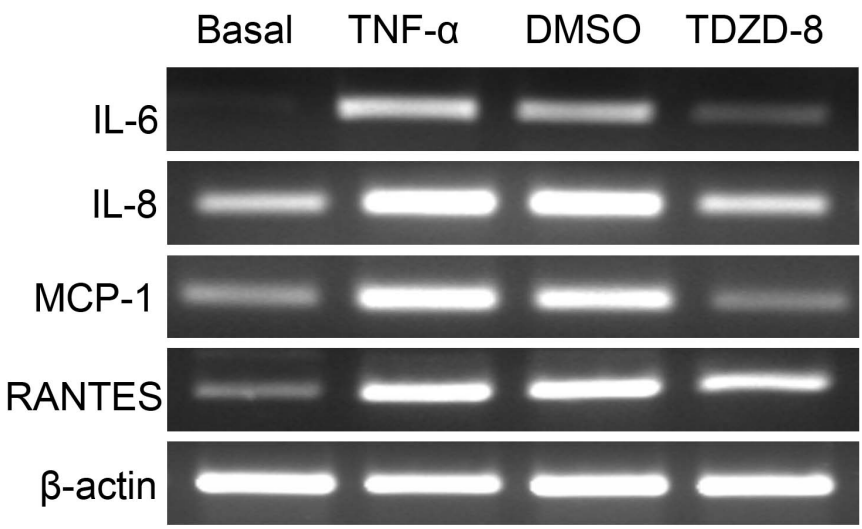


Figure 4.13 Effects of TDZD-8 on TNF-α-induced expressions of proinflammatory cytokines in normal human bronchial epithelial cells. Primary epithelial cells were stimulated with 10 ng/ml TNF-α for 24 hours, with or without pretreatment of 10 μM TDZD-8. Total mRNA was extracted using TriZol reagent and the PCR products were separated in a 2% agarose gel visualized under ultraviolet light. β-actin was used as an internal control. This is a representative gel from three separate experiments with similar pattern of results.

4.2. Discussions

GSK-3β is a serine-threonine kinase that is constitutively active in cells (Cohen and Frame, 2001). Besides its role in glycogen metabolism, GSK-3β also acts as a key regulator in numerous signaling pathways initiated by diverse stimuli. The most well established role of GSK-3β is in the WNT/β-catenin pathway, which is essential for the specification of cell fates during embryogenesis (Huang and He, 2008). In resting cells, GSK3 forms a protein complex with Axin and β-catenin in the cytosol, leading to the phosphorylation and degradation of β-catenin (Ciani and Salinas, 2005). Activation of WNT pathway inhibits GSK3 in the complex, causing the translocation of β-catenin into the nucleus, where it interacts with T-cell factor to initiate the expression of targeted genes, including oncogenes as cycline D1, Myc, and c-jun (Polakis, 1999). Furthermore, mutations in β-catenin, which render it resistant to degradation, are commonly associated with several types of cancers, particularly colon cancer (Lustig and Behrens, 2003). Therefore, one major concern regarding to the long-term use of GSK-3 inhibitors is their contribution to tumorigenesis due to the accumulation of proto-oncogenes. However, no inactivating mutations in GSK3 have been found yet in human cancers, contrasting with the fact that many mutations have been found in human cancers in the other components of WNT pathway (Lustig and Behrens, 2003; Polakis, 1999). Moreover, lithium, the best characterized GSK-3 inhibitor, has been used to treat bipolar diseases for many years, and their long term use has not

been associated with increased risks of cancer (Cohen et al., 1998; Jope, 2003). These findings suggest that inhibition of GSK-3 might not be sufficient to increase the levels of β-catenin in primary cells and induce cancers.

GSK-3β has recently been linked to the regulation of NF-κB transactivation function in a study using GSK-3β knockout mice (Hoeflich et al., 2000). It has been demonstrated that NF-κB activity is up-regulated in allergic airway inflammation both in human and in animal models of asthma (Broide et al., 2005; Donovan et al., 1999; Gagliardo et al., 2003; Hart et al., 1998; Poynter et al., 2004). Various therapeutic strategies targeted at the NF-κB signaling pathway such as NF-κB specific decoy oligonucleotide (Desmet et al., 2004), p65-specific antisense oligonucleotide (Choi et al., 2004), and IKK-2 selective small molecule inhibitors (Birrell et al., 2005) have demonstrated beneficial effects in experimental asthma models. Cumulative evidence shows that GSK-3β can phosphorylate the p65 subunit, particularly the COOH-terminus of p65 at Ser^{536}, to enhance transcriptional responses of NF-κB (De Ketelaere et al., 2004; Hoeflich et al., 2000; Schwabe and Brenner, 2002). Hence, pharmacologic inhibition of GSK-3β may lead to inhibition of NF-κB transcriptional activity and offer the anti-inflammatory potential. Due to the discoveries of both crystal structure and phosphorylation mechanisms of GSK-3β, it is possible to design a specific inhibitor of GSK-3β (Dajani et al., 2001; ter Haar et al., 2001). TDZD-8 is the first highly specific non-ATP competitive GSK-3β inhibitor with micromolar range of

the IC^{50} value and negligible effect against other protein kinases such as protein kinase A, protein kinase C, casein kinase-II, and cyclin-dependent kinase-1 (Martinez et al., 2002; Meijer et al., 2004). Structure activity relationship studies have suggested that the thiadiazolidinone ring in the TDZD-8 compound may be crucial to the inhibition of GSK-3β (Martinez et al., 2002). Therefore, TDZD-8 is a suitable compound for us to study the effects of inhibition of GSK-3β in the allergic inflammation. Our immunoblot analysis revealed a significant inhibition of p65 phosphorylation (Ser^{563}) by TDZD-8 in OVA-challenged lungs and in TNF-α stimulated human bronchial epithelial cells, confirming that inhibition of GSK-3β suppressed NF-κB signaling pathway in inflamed airways and in primary bronchial epithelial cell culture. However, our preliminary study using ELISA to measure lung tissue nuclear extract NF-κB binding activity to DNA revealed that TDZD-8 had no effect on the binding of NF-κB to DNA motif. Taken together, in our mouse asthma model, TDZD-8 attenuated p65 phosphorylation without affecting NF-κB to DNA-binding. Our observation is supported by Hoeflich and colleagues (Hoeflich et al., 2000), Martin and coworkers (Martin et al., 2005), and Dugo and associates (Dugo et al., 2005), but not by Eto and colleagues (Eto et al., 2005). The reason for the observed differences is not clear, but may be related to the disease models used or the doses and pharmacokinetics of the inhibitors employed.

Th2 cells play an essential role in the pathogenesis of the allergic airway inflammation (Herrick and Bottomly, 2003). IL-4, IL-5, and IL-13 can be produced by various resident cells such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages, as well as by infiltrated inflammatory cells such as lymphocytes and eosinophils. NF-κB is a critical transcription factor for Th2 cell differentiation (Das et al., 2001). Our present results show that TDZD-8 significantly reduced the levels of IL-5, IL-13, and eotaxin in BAL fluids from OVA-challenged mice. These findings are consistent with those from OVA-challenged mice with disrupted NF-κB function via conditional knockout of IKKβ or transgenic IκBα mutant expression selectively in airway epithetlium (Broide et al., 2005; Poynter et al., 2004), or via aerosol administration of NF-κB decoy oligonucleotide(Desmet et al., 2004), suggesting that TDZD-8 may exert its anti-inflammatory action on airway epithelium. In addition, antigen receptor activation in T and B cells and in mast cells has been shown to culminate in NF-κB activation (Li et al., 2004; Peng et al., 2005; Weil and Israel, 2004), and TDZD-8 may modulate NF-κB mediated cytokine production in these inflammatory cells. In addition, airway smooth muscle is another source of proinflammatory cytokines during the airway inflammation (Howarth et al., 2004). Repression of NF-κB signaling pathway has been shown to block IL-13 induced eotaxin production in cultured human airway smooth muscle cells (Birrell et al., 2005). As such, the observed reduction of IL-5, IL-13, and eotaxin levels in BAL fluid from GSK-3β

inhibitor TDZD-8 treated mice may be due to inhibition of NF-κB activation in those inflammatory and airway resident cells. Surprisingly, after the treatment of TDZD-8, IL-4 levels in BAL fluid were not affected. However, previous studies also found similar phenomenon which has shown no change in IL-4 levels in BAL fluid in murine models by IκBα mutant mice or by the treatment of NF-κB decoy oligodeoxynucleotides, respectively (Desmet et al., 2004; Poynter et al., 2004). This may be due to the lack of κB site in IL-4 gene or incomplete inhibition of NF-κB pathways, and more studies should be done to elucidate mechanisms. Another possible explanation is the limitation of IL-4 ELISA assay in which there is a minor gap between negative mice and asthmatic mice, and levels of IL-4 in BAL fluid is close to the lowest detection level.

Eosinophils play a central role in the pathogenesis of allergic inflammation (Rothenberg and Hogan, 2006). Our present findings showed that TDZD-8 prevented eosinophil infiltration into the airways as shown by a significant drop in total cell counts and eosinophil counts in BAL fluid, and in tissue eosinophilia in lung sections. Eosinophil transmigration into the airways is a multistep process that is orchestrated by cytokines such as IL-5 and IL-13, and coordinated by specific chemokines such as eotaxin in combination with adhesion molecules such as ICAM-1 and VCAM-1 (Jia et al., 1999; Ono et al., 2003; Rothenberg and Hogan, 2006). IL-13 is by far the most potent inducer of eotaxin expression in airway epithelial cells (Li et al., 1999). We demonstrated that TDZD-8 slightly

down-regulated the expression of VCAM-1 and ICAM-1 mRNA in OVA-challenged lungs. These findings are likely to be due to TDZD-8–mediated NF-κB inhibition, as genes for VCAM-1 and ICAM-1 contain the κB site for NF-κB within their promoters (Holden et al., 2004; Kumar et al., 2004). Taken together, the observed reduction in airway eosinophilia by GSK-3β inhibitor TDZD-8 may be a result of composite inhibitory effects on IL-13 and eotaxin production, and on VCAM-1 and ICAM-1 expression, secondary to inhibition of NF-κB activation.

We have also demonstrated a dramatic reduction in mucus production in TDZD-8–treated mice as compared with DMSO control. Cumulative evidence indicates that IL-5 and IL-13 play a critical role in goblet cell hyperplasia and mucin Muc5ac gene and protein expression in mice (Justice et al., 2002; Zuhdi Alimam et al., 2000). Interestingly, Muc5ac gene expression is dependent on the transcriptional activity of NF-κB (Lora et al., 2005), and selective ablation of NF-κB function in airway epithelium blocked OVA-induced mucus production in mice (Broide et al., 2005). We also observed a dramatic drop in Muc5ac mRNA expression by TDZD-8 in OVA-challenged lungs. As such, the marked decrease in mucus production in TDZD-8 treated lungs may be attributed to a substantial drop in IL-5 and IL-13 levels, and a direct inhibitory action on NF-κB in airway epithelium in asthmatic mice treated with the GSK-3β inhibitor. Elevated serum IgE levels are a hallmark of the Th2 immune response. Our data showed that serum levels of OVA-specific IgE were substantially reduced by TDZD-8. NF-κB

plays a crucial role in B cell receptor–induced proliferation of mature B cells (Li et al., 2004; Weil and Israel, 2004). In addition, IL-13 is important in directing B cell growth, differentiation, and secretion of IgE (Emson et al., 1998; Wills-Karp, 2004). The biological activities of IgE are mediated through its interaction with FcεRI on mast cells and basophils. Cross-linking of FcεRI initiates multiple signaling cascades leading to NF-κB activation and production of cytokines and chemokines (Lorentz et al., 2003; Peng et al., 2005). Therefore, the observed reduction in serum OVA-specific IgE by TDZD-8 in our asthma model may be contributed by its inhibitory effects on B cell activation and on IL-13 production by inflammatory cells such as mast cells, secondary to inhibition of NF-κB activation.

A family of chitinase proteins including AMCase, Ym1, and Ym2 has recently been found to be markedly elevated in allergic airway inflammation and to play a role in the pathogenesis of asthma (Zhao et al., 2005; Zhu et al., 2004). They are mainly expressed in airway epithelium and alveolar macrophages. A recent study demonstrated an increase in AMCase levels in a mouse asthma model and in subjects with asthma in an IL-13 dependent manner (Zhu et al., 2004). When given intratracheally, IL-13 elevated Ym1 and Ym2 levels in BAL fluid from mice in vivo (Webb et al., 2001). Although the molecular mechanisms of action of chitinases in asthma are largely unknown, they can specifically bind to carbohydrate moieties such as GlcN oligomers and other glycosaminoglycans such

as heparin and heparan sulfate to regulate eosinophil chemotaxis, inflammation, and tissue remodeling (Elias et al., 2005; Zhu et al., 2004). Our data show that TDZD-8 slightly down-regulated AMCase, moderately inhibited Ym1, but dramatically suppressed Ym2 mRNA levels in OVA-challenged lungs. The reduction of chitinases, especially Ym2, by TDZD-8 may be a result of the major drop in IL-13 level in the airways (of animals treated with TDZD-8) and contribute to the diminished pulmonary eosinophilia.

It is believed that inflammatory mediators released during the allergic inflammation play a critical role in AHR development (Cohn et al., 2004; Fernandes et al., 2003). We report here that TDZD-8 significantly inhibited OVA-induced AHR to increasing concentrations of methacholine. It has been established that IL-5 plays a critical role in AHR by mobilizing and activating esoinophils, leading to the release of pro-inflammatory products such as major basic protein and cysteinyl-leukotrienes, which are closely associated with AHR (Rothenberg and Hogan, 2006). In addition, IL-13 has been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated in AHR (Vargaftig and Singer, 2003; Wills-Karp, 2004). Moreover, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (Lorentz et al., 2003). Thus, the observed reduction of AHR by GSK-3β inhibition may be associated with reduction in Th2 cytokine production, tissue eosinophilia, and serum IgE level by TDZD-8.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report here for the first time that GSK-3β inhibition effectively reduced OVA-induced IL-5, IL-13 and eotaxin production, pulmonary eosinophilia, serum IgE synthesis, mucus hypersecretion, and AHR in a mouse asthma model. These findings support a potential role for GSK-3β inhibitor in the treatment of asthma via the inhibition of NF-κB p65 phosphorylation.

However, there are still many discrepancies between asthma patients and mouse models which are difficult to reconcile. For example, asthmatic mice exhibit only transient mechacholine-induced AHR, whereas asthma patients show signs of both transient and consistent AHR. The asthma model used in this study is an acute mouse model, while most of asthma patients are induced by chronic allergen exposure. Therefore, it remains uncertain whether it is a potential and useful therapy for human patients. More studies confirming the known effects of TDZD-8 in the acute asthma model need to be done in other models.

5. ANTI-INFLAMMATORY EFFECTS OF ANDROGRAPHOLIDE IN A MOUSE ASTHMA MODEL

5.1. Results

5.1.1. Effects of andrographolide on OVA-induced inflammatory cell recruitment in BAL fluid

To determine the effects of andrographolide on the OVA-induced airway inflammation, BAL fluid was collected 24 hours after the last OVA or saline aerosol challenge, and total and differential cell counts were performed. In saline-challenged mice, almost all BAL fluid cells were macrophages. OVA inhalation significantly ($P < 0.05$) increased total cell, eosinophil, lymphocyte, and neutrophil counts as compared with saline control (Figure 5.1). DMSO (2%) as vehicle did not show any inhibitory effects on BAL fluid cell counts in mice challenged with OVA (Figure 5.2). Our experience in using lower percentage of DMSO resulted in the precipitation of andrographolide and subsequent high variability in experimental endpoint. Andrographolide (0.1, 0.5 and 1 mg/kg) substantially decreased the total cell and eosinophil counts in BAL fluid in a dose-dependent manner as compared with the DMSO vehicle control (Figure 5.2). Andrographolide at high dose (1 mg/kg) also reduced macrophage, lymphocyte, and neutrophil counts (Figure 5.2). We have used 5 mg/kg andrographolide in our preliminary study. Nevertheless, the inhibitory effects observed were no greater than 1 mg/kg andrographolide. As such, the highest dose used in the present study was determined to be 1 mg/kg.

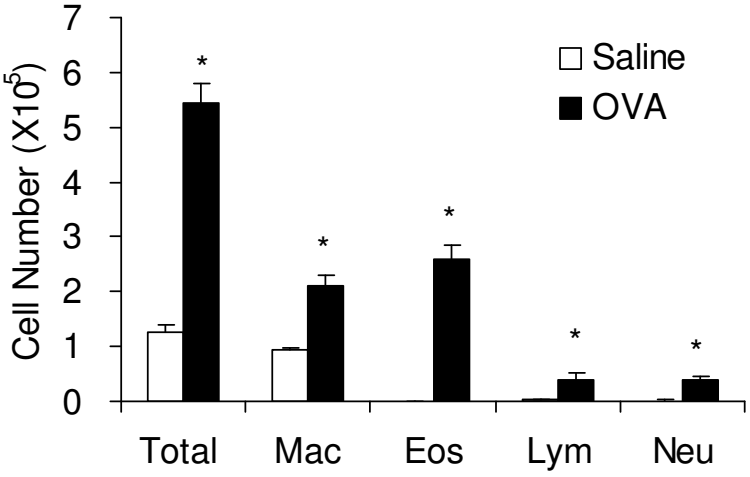


Figure 5.1 Differential cell counts in BAL fluid. Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 hours after the last saline aerosol or 10 mg/ml OVA aerosol challenge (saline, n = 6; OVA, n = 7). Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). *Significant difference from DMSO control, $P < 0.05$.

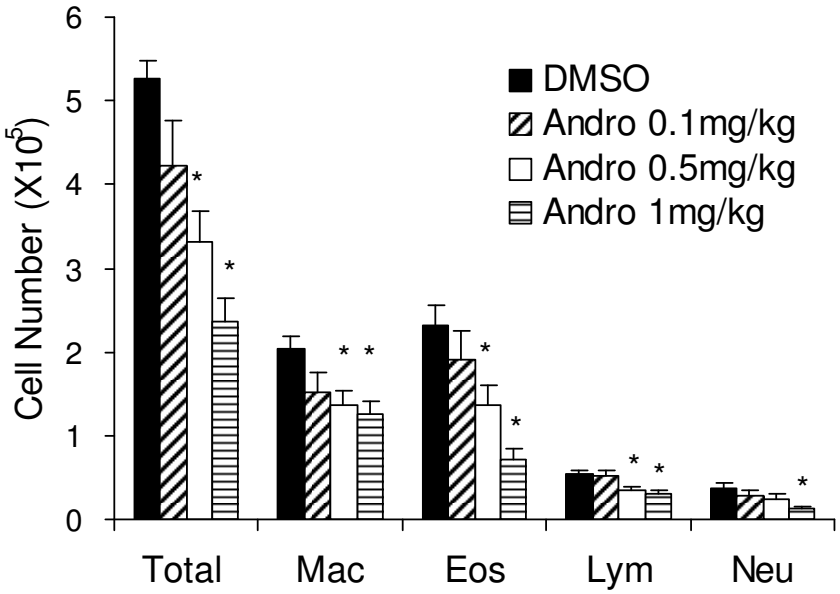


Figure 5.2 Effects of andrographolide on BAL fluid cell infiltration. Andrographolide reduced OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 hours after the last 10 mg/ml OVA aerosol challenge (DMSO, n = 7; 0.1 mg/kg, n = 7; 0.5 mg/kg, n = 10; 1 mg/kg, n = 9). Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). *Significant difference from DMSO control, $P < 0.05$.

To further investigate if this inhibition of inflammatory cell migration into airway is due to the non-specific cytotoxic effects of andrographolide, we tested the population of peripheral blood leukocytes in mice. Flow cytometric analysis revealed that peripheral blood leukocytes from saline-challenged, OVA-challenged, vehicle control, and andrographolide-treated mice had similar percentages of CD3+, CD4+, CD8+ T cells, B cells (B220), and NK cells (NK 1.1) (Figure 5.3). Hence, these results clearly demonstrate that andrographolide inhibits the recruitment of inflammatory cells during airway allergic inflammation.

5.1.2. Effects of andrographolide on OVA-induced airway cell infiltration and mucus production

To investigate effects of andrographolide on inflammatory cell infiltration into peribronchiolar and perivascular regions of the airway, as well as the mucus production, lung tissue was collected 24 hours after the last OVA or saline challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared to saline challenge (Figure 5.4 A and B). Andrographolide (1 mg/kg) markedly diminished the eosinophil-rich leukocyte infiltration as compared to DMSO control (Figure 5.4 C, D, and I). On the other hand, OVA-challenged mice, but not saline-challenged mice, developed marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung (Figure 5.4 E and F).

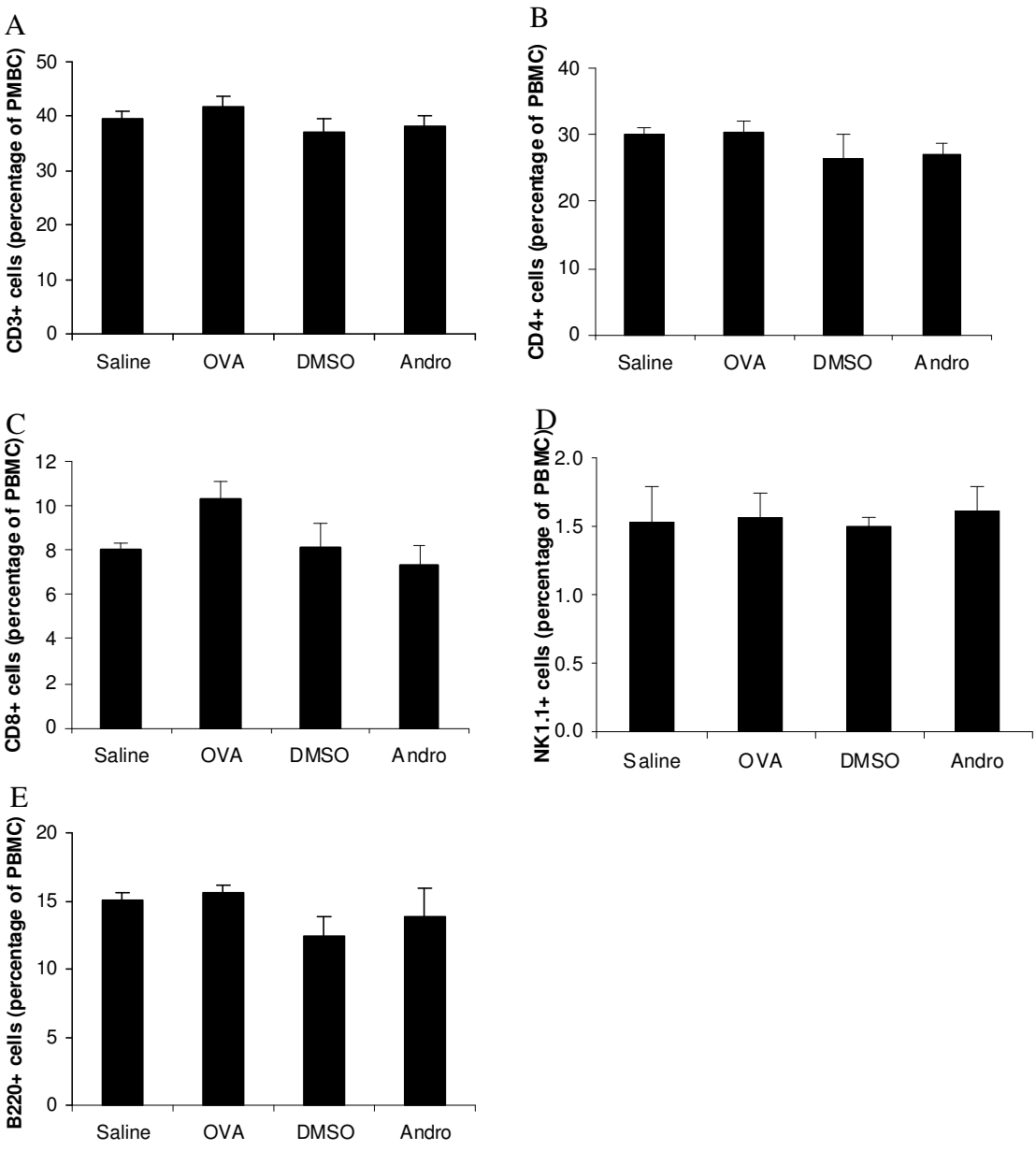
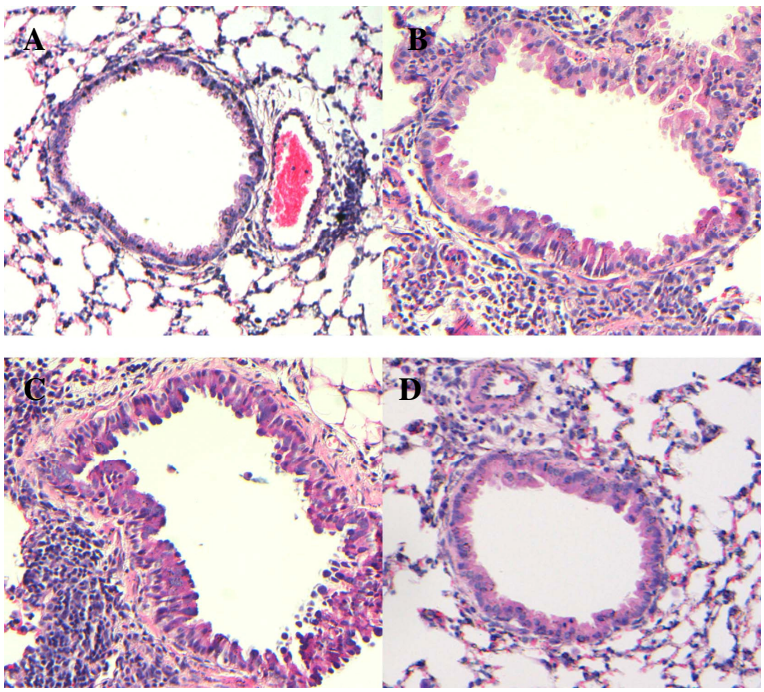


Figure 5.3 Effects of andrographolide on PBMC. Plasma was collected 24 hours after the last OVA or saline aerosol challenge. Expressions of (A) CD3, (B) CD4, (C) CD8, (D) NK1.1 and (E) B220 on PBMC were analyzed using FACS (n = 3 - 5 mice per group). The dose of andrographolide was 1 mg/kg. Values shown are the mean ± SEM. No Significant difference from DMSO control, $P > 0.05$.



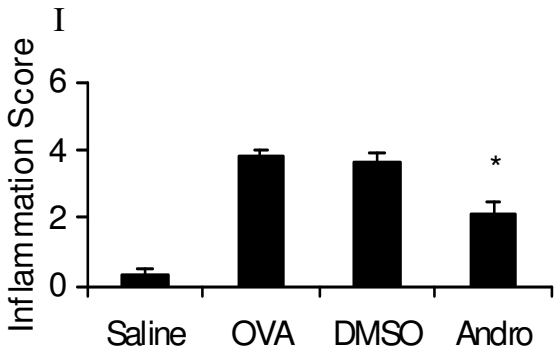


Figure 5.4 A-D, I Effects of andrographolide on lung tissue inflammatory cell infiltration. Histologic examination of lung tissue eosinophilia (A, B, C, and D, magnification ×200) 24 hours after the last challenge of saline aerosol (A), OVA aerosol (B), OVA aerosol plus DMSO (C), or OVA aerosol plus 1 mg/kg andrographolide (D). Quantitative analyses of inflammatory cell infiltration (I) in lung sections were performed as described in chapter 3. Scoring of inflammatory cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $P < 0.05$.

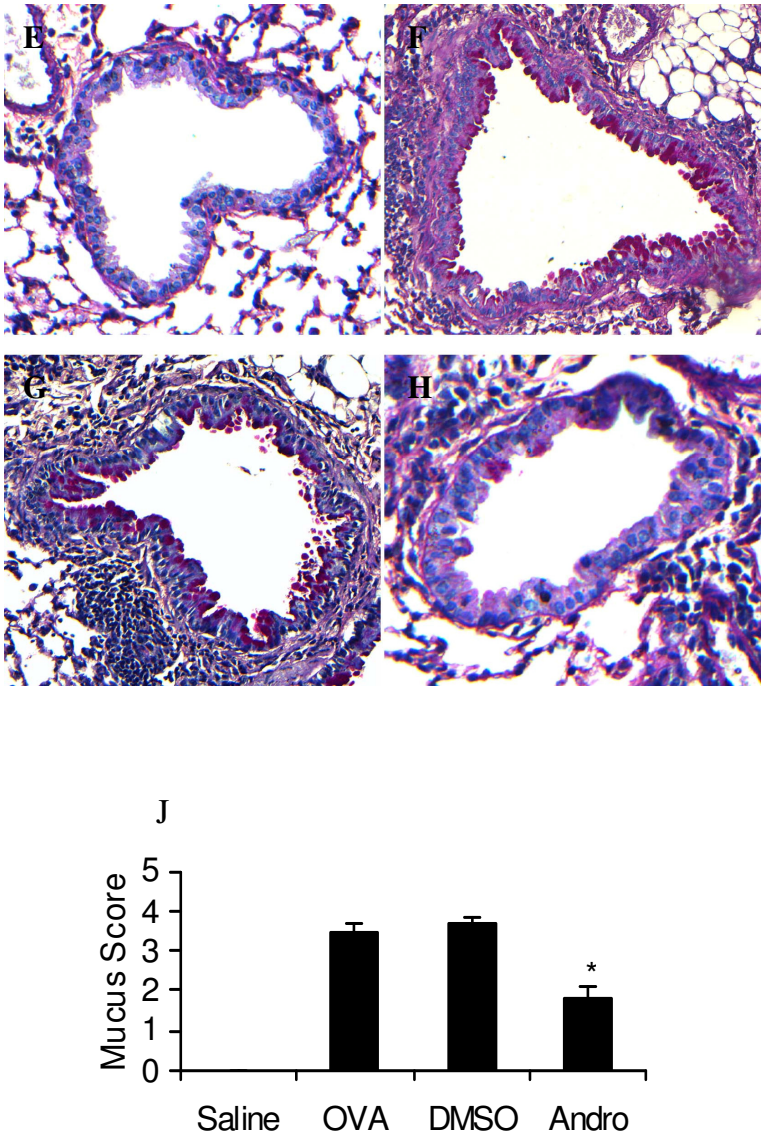


Figure 5.4 E-H, J Effects of andrographolide on mucus production. Histologic examination of lung mucus secretion (E, F, G, and H, magnification ×200) 24 hours after the last challenge of saline aerosol (E), OVA aerosol (F), OVA aerosol plus DMSO (G), or OVA aerosol plus 1 mg/kg andrographolide (H). Quantitative analyses of mucus production (J) in lung sections were performed as described in chapter 3. Scoring of goblet cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $P < 0.05$.

The OVA-induced mucus secretion was significantly reduced by andrographolide (1 mg/kg) when compared to the DMSO control (Figure 5.4 G, H, and J). DMSO has no effects on both the inflammatory cell infiltration and mucus hypersecretion induced by OVA (Figure 5.4 C and G).

5.1.3. Effects of andrographolide on cytokine levels in BAL fluid

To determine the inflammatory cytokine levels in the lung, BAL fluid levels of IL-4, IL-5, IL-13, eotaxin, and IFNγ were measured using ELISA. OVA challenge in sensitized mice substantially increased IL-4, IL-5, and IL-13 levels into BAL fluid as compared with saline control (Figure 5.5). OVA moderately increased BAL fluid eotaxin level (Figure 5.6). In contrast, IFNγ, the Th1 cytokine, was slightly decreased in OVA-challenged mice (Figure 5.7). Andrographolide significantly ($P < 0.05$) decreased IL-4, IL-5, and IL-13 in a dose dependant manner (Figure 5.5). At 1 mg/kg, andrographolide significantly reduced eotaxin levels in BAL fluid as compared with DMSO control (Figure 5.6). Interestingly, andrographolide (1mg/kg) markedly up-regulated IFNγ levels (Figure 5.7), shifting the Th-2 dominant animal model to Th1 immune response. The change of cytokine profile in BAL fluid after the treatment of andrographolide indicates that this drug inhibits an OVA-specific Th2 response.

5.1.4. Effects of andrographolide on serum Ig levels

In order to evaluate whether andrographolide could modify an ongoing OVA-specific Th2 response in vivo,

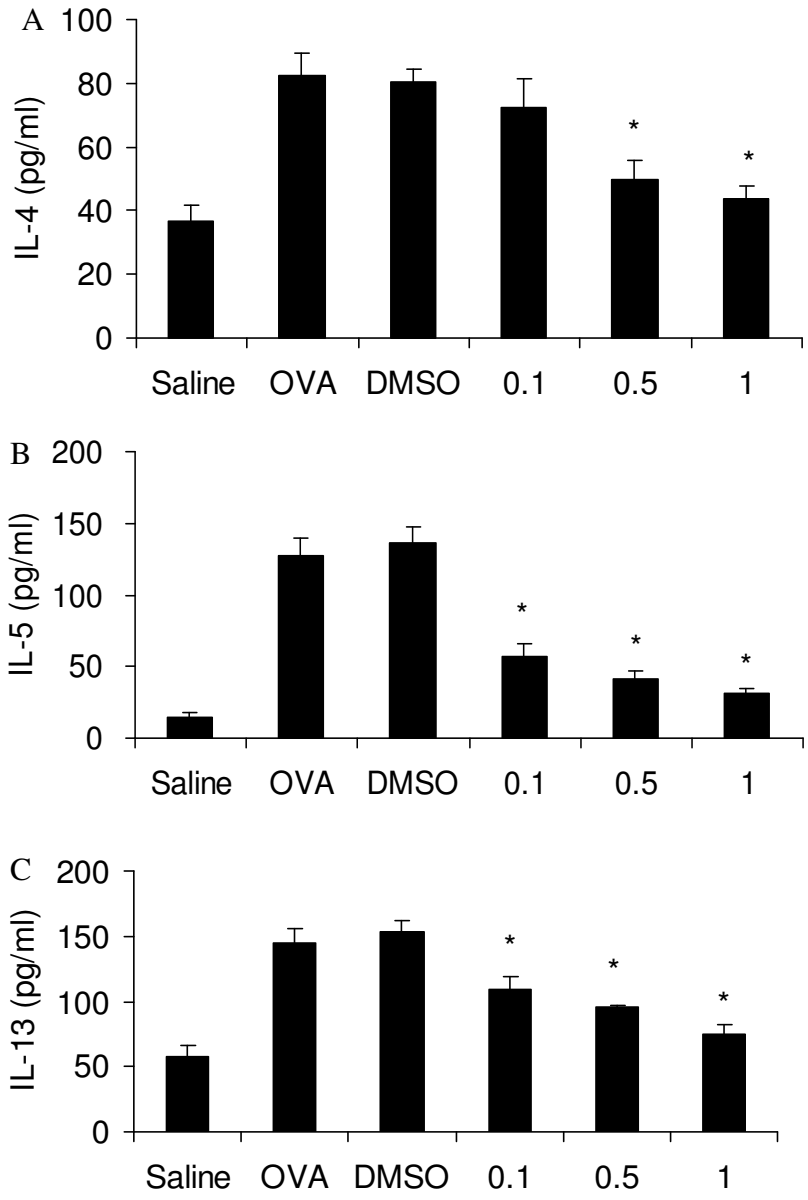


Figure 5.5 Effects of andrographolide on Th2 cytokines in BAL fluid. BAL fluids were collected 24 hours after the last OVA or saline aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 0.1, 0.5, and 1 group indicates asthmatic mice which were treated with three different doses of andrographolide (0.1 = 0.1 mg/kg, 0.5 = 0.5 mg/kg, and 1 = 1 mg/kg). Levels of (A) IL-4, (B) IL-5, and (C) IL-13 were analyzed using ELISA (n = 6 - 9 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

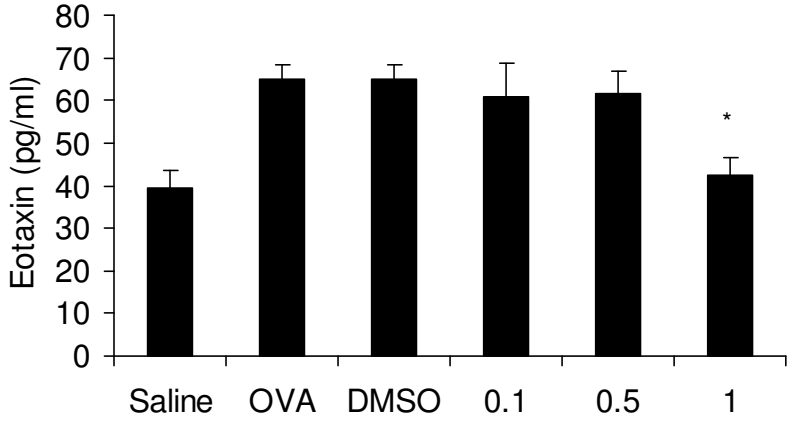


Figure 5.6 Effects of andrographolide on eotaxin level in BAL fluid. BAL fluids were collected 24 hours after the last OVA or saline aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 0.1, 0.5, and 1 group indicates asthmatic mice which were treated with three different doses of andrographolide (0.1 = 0.1 mg/kg, 0.5 = 0.5 mg/kg, and 1 = 1 mg/kg). Level of eotaxin was analyzed using ELISA (n = 6 - 9 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

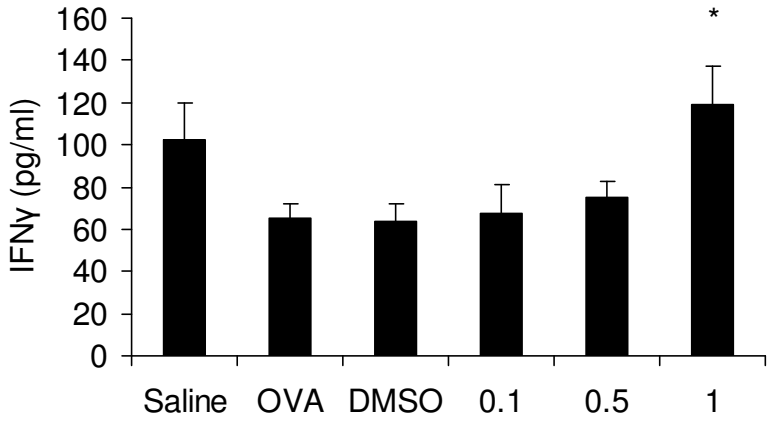


Figure 5.7 Effects of andrographolide on IFNγ level in BAL fluid. BAL fluids were collected 24 hours after the last OVA or saline aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 0.1, 0.5, and 1 group indicates asthmatic mice which were treated with three different doses of andrographolide (0.1 = 0.1 mg/kg, 0.5 = 0.5 mg/kg, and 1 = 1 mg/kg). Level of IFNγ was analyzed using ELISA (n = 6 - 9 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

serum levels of total IgE, OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a were determined using ELISA. Substantial elevations in total IgE, OVA-specific IgE, and OVA-specific IgG1, but not in OVA-specific IgG2a, were observed in serum from OVA sensitized and challenged mice when compared to saline-challenged mice (Figure 5.8 and Figure 5.9). Surprisingly, andrographolide was able to significantly ($P < 0.05$) inhibit OVA-specific IgE levels, even at the lowest dose (0.1 mg/kg) (Figure 5.8), whereas lowered the level of total IgE and OVA-specific IgG1 in a dose dependent manner (Figure 5.8 and Figure 5.9). Andrographolide had no effects on the level of OVA-specific IgG2a (Figure 5.9), indicating a specific inhibition of Th2 response by andrographolide.

5.1.5. Effects of andrographolide on antigen recall in bronchial lymph node cells

To assess whether andrographolide directly affect OVA-specific immune responses in lymphocytes, bronchial lymph nodes (3 - 4 nodes per mouse) were isolated and cultured, 24 hours after the last OVA or saline aerosol challenge, with or without andrographolide pre-treatment. Afterwards, single-cell preparations from lymph nodes were treated with OVA (200 μg/ml) for 72 hours, supernatant levels of IL-4, IL-5, and IFNγ were measured by ELISA. The in vitro OVA-specific production of IL-4 and IL-5 was markedly higher in lymphocytes isolated from OVA-challenged mice than those from saline-challenged mice (Figure 5.10 A and B).

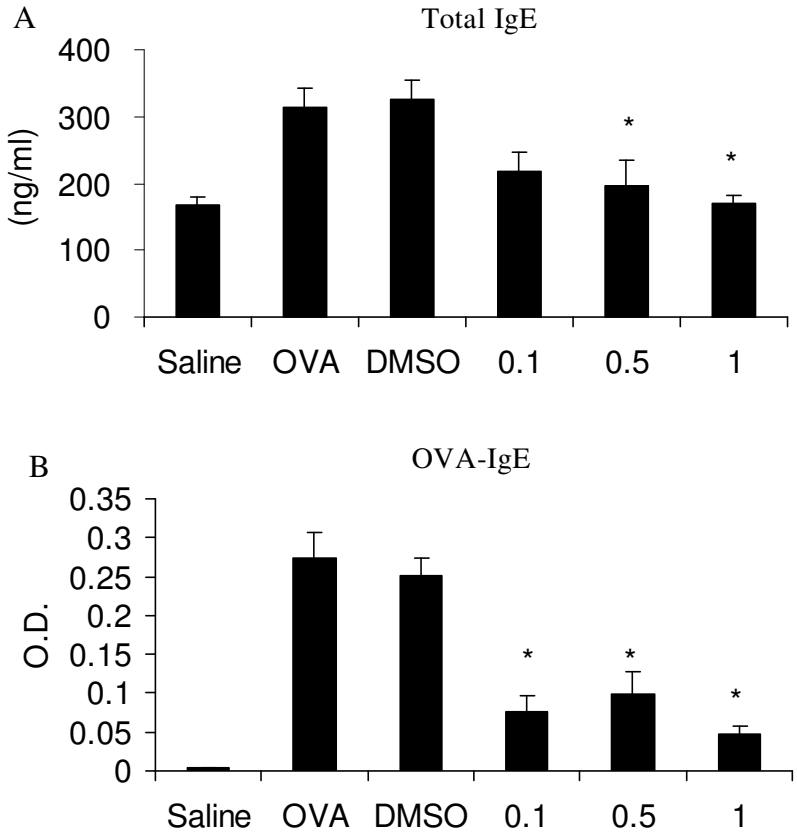


Figure 5.8 Effects of andrographolide on serum IgE production. Mouse serum was collected 24 hours after the last OVA or saline aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 0.1, 0.5, and 1 group indicates asthmatic mice which were treated with three different doses of andrographolide (0.1 = 0.1 mg/kg, 0.5 = 0.5 mg/kg, and 1 = 1 mg/kg). The levels of (A) total IgE and (B) OVA-specific IgE were analyzed using ELISA (n = 6 – 9 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

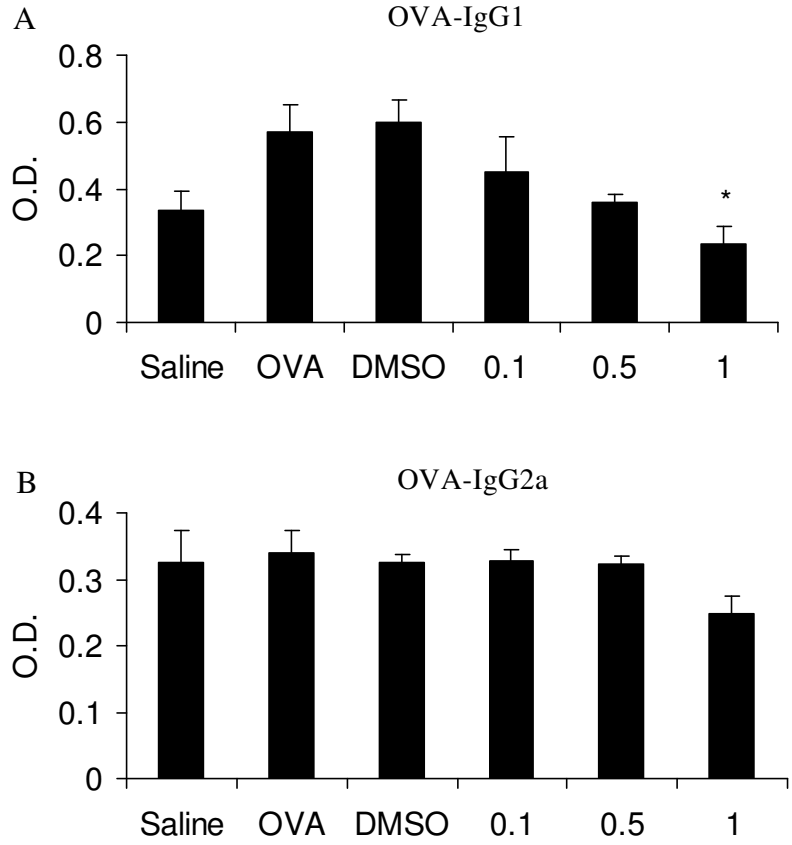


Figure 5.9 Effects of andrographolide on serum IgG production. Mouse serum was collected 24 hours after the last OVA or saline aerosol challenge. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 0.1, 0.5, and 1 group indicates asthmatic mice which were treated with three different doses of andrographolide (0.1 = 0.1 mg/kg, 0.5 = 0.5 mg/kg, and 1 = 1 mg/kg). The levels of (A) OVA-specific IgG1 and (B) OVA-specific IgG2a were analyzed using ELISA (n = 6 – 9 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

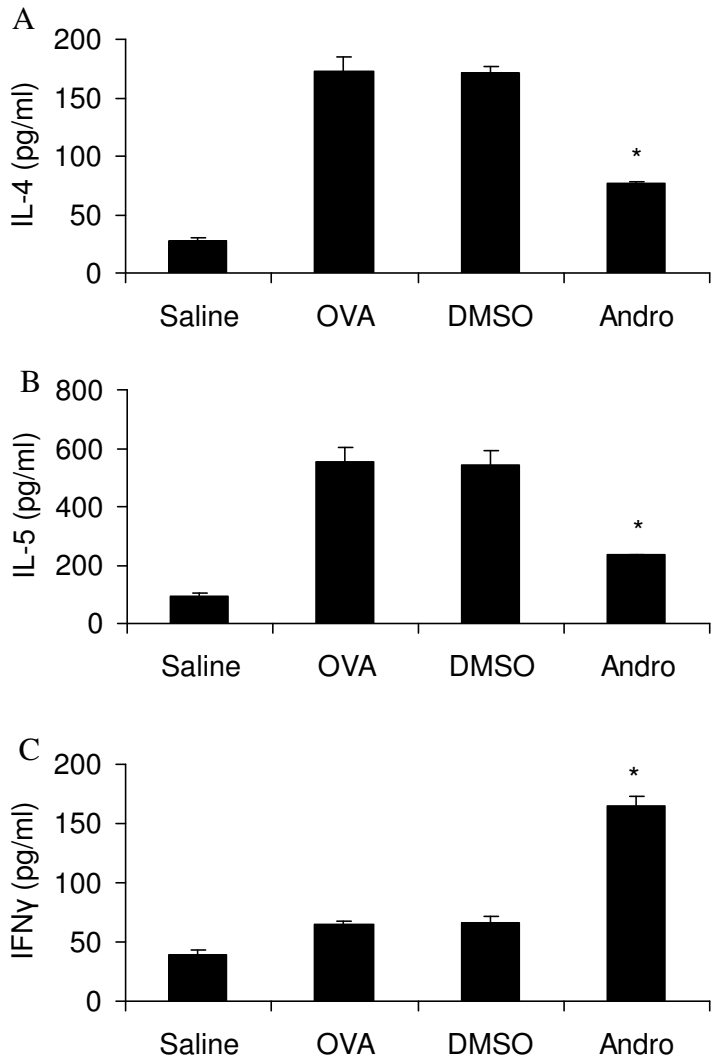


Figure 5.10 Effects of andrographolide on OVA-specific response in vitro. Thoracic lymph nodes cells (n = 3 mice per group) were harvested from mice 24 hours after the last OVA or saline aerosol challenge and cultured for 72 hours with OVA. The saline group refers to mice which were sensitized with OVA and challenged with saline. The OVA group refers to asthmatic mice which were both sensitized and challenged with OVA. The DMSO group indicates asthmatic mice which were treated with the drug solvent, DMSO. The 0.1, 0.5, and 1 group indicates asthmatic mice which were treated with three different doses of andrographolide (0.1 = 0.1 mg/kg, 0.5 = 0.5 mg/kg, and 1 = 1 mg/kg). The levels of (A) IL-4, (B) IL-5, and (C) IFNγ were analyzed using ELISA (n = 3 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

Andrographolide (1 mg/kg) pre-treatment significantly ($P < 0.05$) lowered the levels of both IL-4 and IL-5 (Figure 5.10 A and B). In addition, OVA-specific IFNγ production was found to be increased in mice treated with andrographolide (1 mg/kg) (Figure 5.10 C). Finally immune modulation by andrographolide in vivo was OVA-specific because concanavalin A (Con-A) induced production of IL-4, IL-5, and IFNγ in parallel cultures was not affected (Figure 5.11 A, B, and C).

5.1.6. Effects of andrographolide on lung mRNA expression of inflammatory markers

To determine what other inflammatory mediators are affected by andrographolide, lung tissues were obtained 24 hours after the last OVA or saline challenge and processed to study mRNA levels of target mediators by RT-PCR. Recruitment of leukocytes from vessels into inflammatory sites are essential for the initiation of inflammatory responses to the development of asthma (Kelly et al., 2007). This recruitment of leukocytes, particularly eosinophils, is mediated by adhesion molecules (Jia et al., 1999; Medoff et al., 2008). OVA aerosol challenge moderately up-regulated the mRNA levels of adhesion molecules ICAM-1, VCAM-1, and E-selectin (Figure 5.12). Recent studies have revealed that a family of chitinase proteins including AMCase, Ym1, Ym2, and YKL-40, play critical roles in airway inflammation and remodeling (Moffatt and Cookson, 2008; Ober et al., 2008; Webb et al., 2001; Zhao et al., 2005; Zhu et al., 2004).

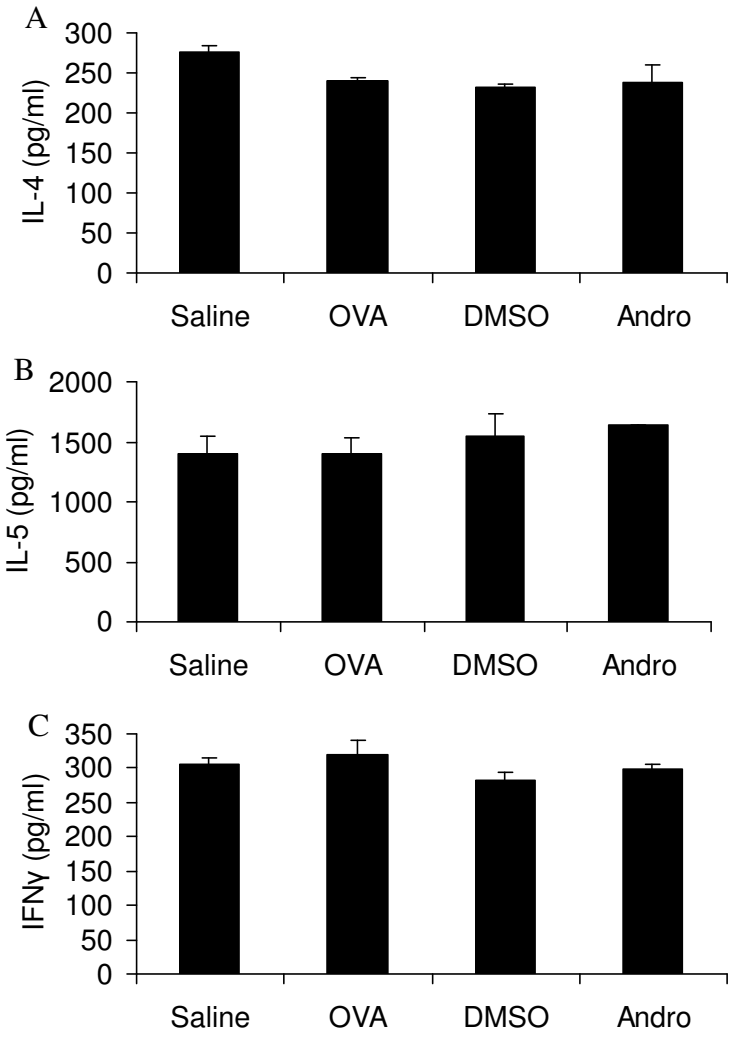


Figure 5.11 Effects of andrographolide on Con-A response in vitro. Thoracic lymph nodes cells (n = 3 mice per group) were harvested from mice 24 hours after the last OVA or saline aerosol challenge and cultured for 72 hours with Con-A. The levels of (A) IL-4, (B) IL-5, and (C) IFNγ were analyzed using ELISA (n = 3 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

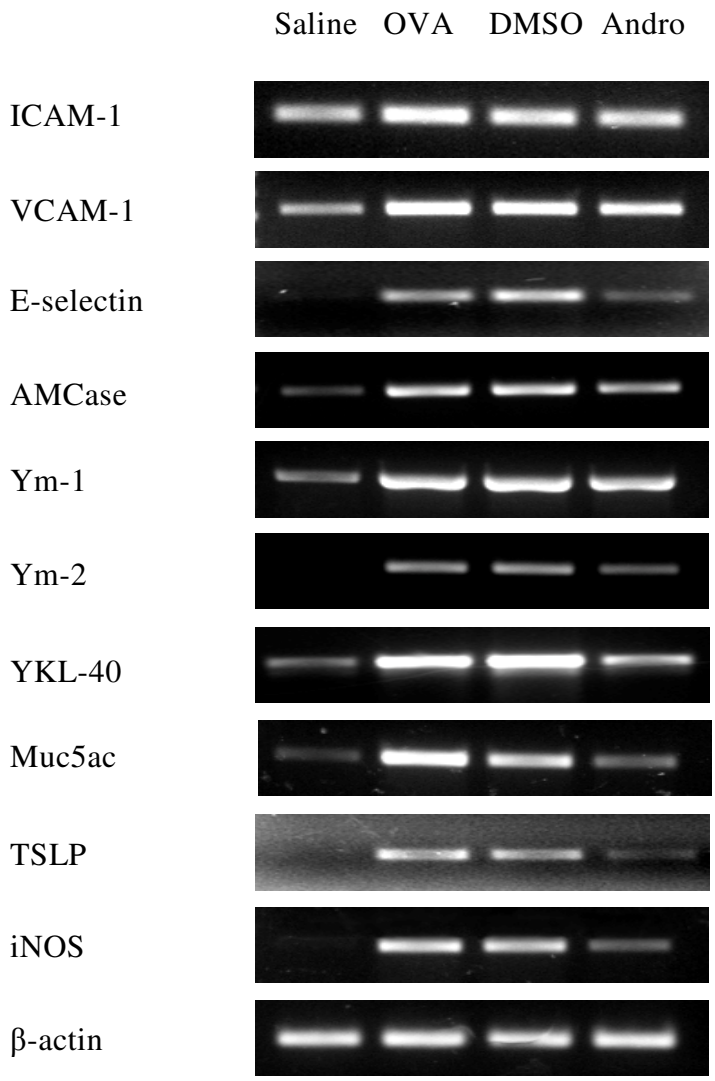


Figure 5.12 Effects of andrographolide on pulmonary mRNA expression of inflammatory markers. Lung tissues were collected 24 hours after the last OVA aerosol challenge. Total mRNA was extracted using TriZol reagent and the PCR products were separated in a 2% agarose gel visualized under ultraviolet light. β-actin was used as an internal control. The experiments were repeated for three times (n = 3 mice per group) with similar pattern of results.

All of these chitinase proteins were found to be elevated in our mouse asthma model (Figure 5.12).Mucus hypersecretion is the hallmark of asthama and Muc5ac is essential for mucin production (Zuhdi Alimam et al., 2000). TSLP, which is produced by epithelial cells, has been found to condition DC maturation and regulate Th2 responses in asthma (Liu et al., 2007; Soumelis et al., 2002). Nitric oxide (NO) is a mediator of airway and smooth muscle tone, which is synthesized by enzymes that have cytokine inducible form such as inducible nitric oxide synthase (iNOS) (Fischer et al., 2002). iNOS is up-regulated in the airways of asthmatic patients (Hamid et al., 1993). OVA challenge dramatically increased the expression of Muc5ac, TSLP, and iNOS (Figure 5.12). We found that pre-treatment with andrographolide (10 mg/kg) moderately decreased levels of ICAM-1, VCAM-1, and dramatically reduced the level of E-selectin, which is consistent with the decreased levels of leukocytes in BAL fluid (Figure 5.12). Furthermore, andrographolide reduced gene expressions of inflammatory mediators such as Chitinases, Muc5ac, TSLP, iNOS in the allergic airway (Figure 5.12).

5.1.7. Effects of andrographolide on OVA-induced AHR in mice

To investigate the effect of andrographolide on AHR in asthmatic mice in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice with and without pretreatment of andrographolide. As compared to saline-challenged group, OVA-challenged mice

developed greater AHR which is typically reflected by high RI and low Cdyn (Figure 5.13 and Figure 5.14). Andrographolide (1 mg/kg) dramatically suppressed RI (Figure 5.13) and restored Cdyn (Figure 5.14) in OVA-challenged mice in response to methacholine as compared to DMSO control, suggesting that OVA-induced AHR was modified after the treatment of andrographolide. DMSO did not affect AHR in OVA-challenged mice (Figure 5.13 and Figure 5.14).

5.1.8. Effects of andrographolide on TNF-α-induced NF-κB activation in A549 cells

To explore anti-inflammatory mechanisms of andrographolide, we studied the effects of andrographolide on TNF-α induced NF-κB activation in A549 cells. A549 cells were pre-incubated with andrographolide (30 μM) for 4 hours and then exposed to TNF-α (10 ng/ml). Total proteins of cells were harvested at 0, 5, 15, and 30 min after the stimulation of TNF-α. TNF-α quickly induced the phosphorylation of IKKβ and IκBα within 5 min, leading to the following degradation of IκBα (Figure 5.15). Andrographolide completely inhibited the phosphorylation of IKKβ, markedly reduced the phosphorylation of IκBα, and delayed the following IκBα degradation as compared with control (Figure 5.15). As one of p65 kinases, IKKβ can phosphorylate p65 at Ser^{536} and enhance the transcriptional activity of p65 (Perkins, 2007). Andrographolide also decreased the TNF-α-induced phosphorylation of p65 as compared with control (Figure 5.15).

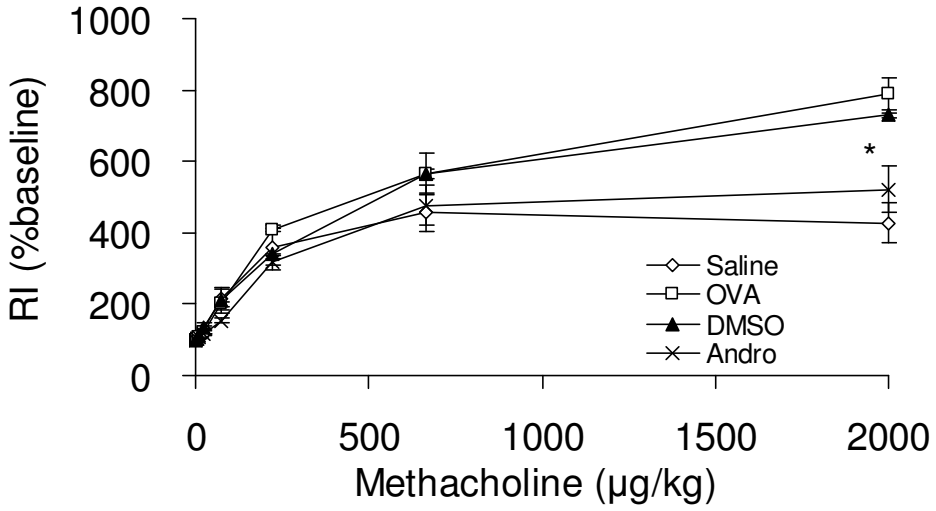


Figure 5.13 Effects of andrographolide on airway resistance. Airway responsiveness of mechanically ventilated mice in response to intravenous methacholine was measured 24 hours after the last saline aerosol or OVA aerosol with pretreatment of either DMSO or 1 mg/kg andrographolide. AHR is expressed as percentage change from the baseline level of RI (n = 5). *Significant difference from DMSO control, $P < 0.05$.

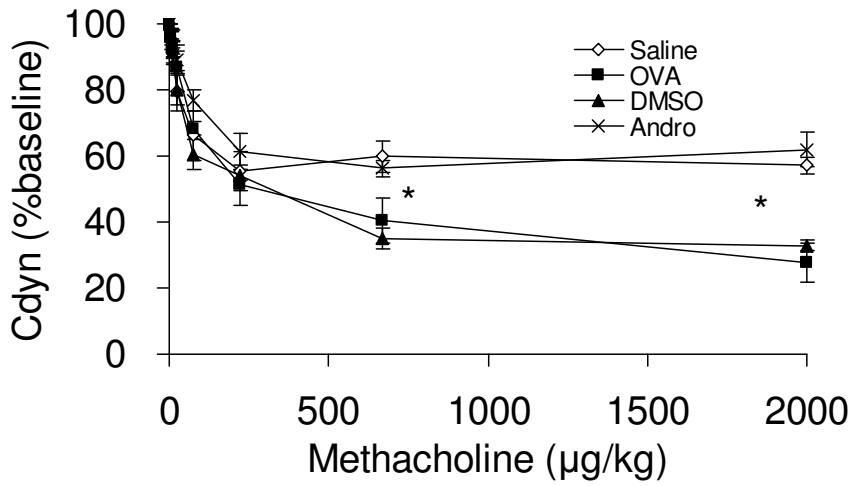


Figure 5.14 Effects of andrographolide on airway dynamic compliance. Airway responsiveness of mechanically ventilated mice in response to intravenous methacholine was measured 24 hours after the last saline aerosol or OVA aerosol with pretreatment of either DMSO or 1 mg/kg andrographolide. AHR is expressed as percentage change from the baseline level of Cdyn (n = 5). *Significant difference from DMSO control, $P < 0.05$.

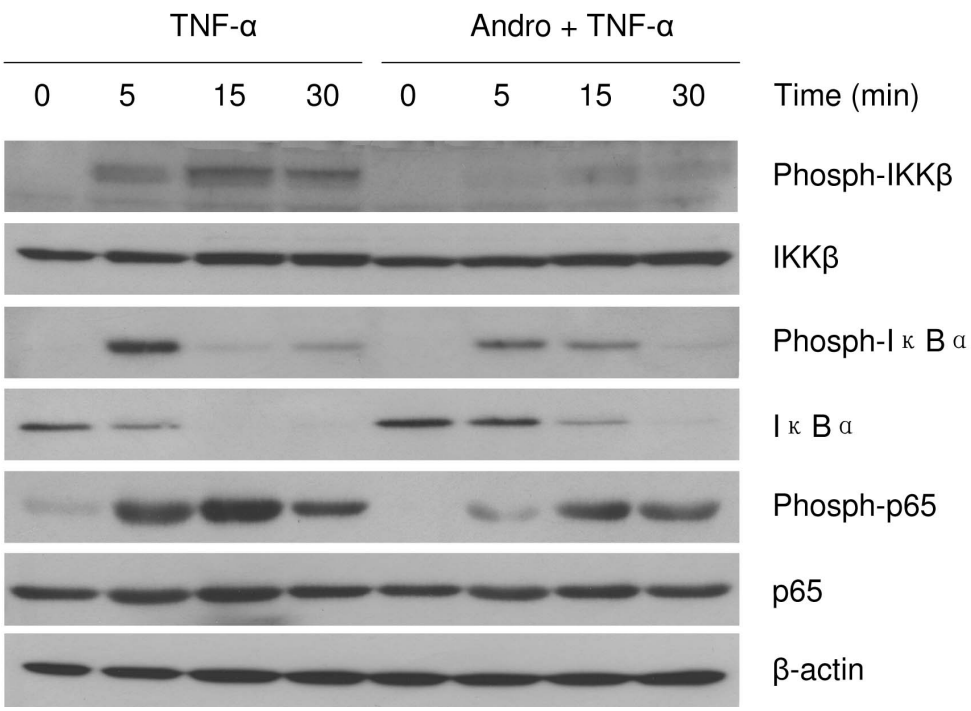


Figure 5.15 Effects of andrographolide on TNF-α induced NF-κB activation in A549 cells. A549 cells were incubated with or without andrographolide (30 μM) for 4 hours and then stimulated with TNF-α (10 ng/ml) for the indicated times. Total proteins of cells were isolated and analyzed by Western blotting with the indicated antibodies. β-actin was used as an internal control. The experiments were repeated for three times with similar pattern of results.

Because IκBα degradation is required for the activation of NF-κB canonical pathway, we further tested the translocation of p65 and p65 DNA-binding activity using nuclear proteins isolated from the cells, 30 min after the stimulation of TNF-α. TNF-α induced the accumulation of p65 on nucleus (Figure 5.16) and increased the p65 DNA-binding activity (Figure 5.17). Andrographolide reduced the translocation of p65 and DNA-binding activity as compared with control (Figure 5.16 and Figure 5.17), suggesting andrographolide reduces the activity of the NF-κB pathway via inhibiting the phosphorylation of IKKβ.

5.1.9. Effect of andrographolide on NF-κB DNA-binding activity in vivo

To study if andrographolide modulates NF-κB activity in the asthma model, lung tissues were obtained 24 hours after the last OVA or saline challenge and a TransAM assay was performed on the nuclear proteins to measure NF-κB DNA-binding activity. OVA challenge markedly increased p65 DNA-binding activity in the lung tissue as compared with saline control, indicating enhanced NF-κB activity in asthma model (Figure 5.18). Andrographolide was able to significantly ($P < 0.05$) reduce DNA-binding activity when compared with DMSO vehicle control, suggesting andrographolide could decrease NF-κB activity and block inflammatory responses in asthma (Figure 5.18). DMSO had no effects on DNA-binding activity as compared with OVA-challenged group. The NF-κB signaling pathway is essential in both innate and adaptive immune responses and in the regulation of apoptosis.

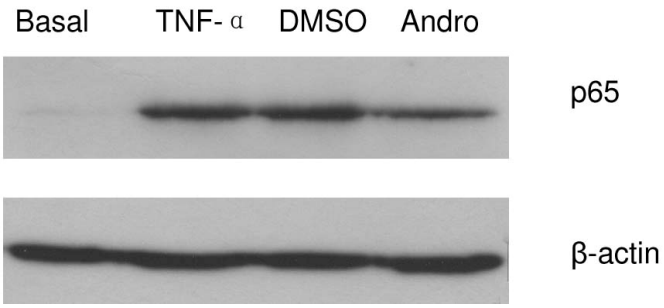


Figure 5.16 Effects of andrographolide on TNF-α induced translocation of p65 in A549 cells. A549 cells were incubated with or without andrographolide (30 μM) for 4 hours and then stimulated with TNF-α (10 ng/ml) for 30 min. Nuclear proteins of cells were isolated and analyzed by Western Blotting with p65 antibody. β-actin was used as an internal control. The experiments were repeated for three times with similar pattern of results.

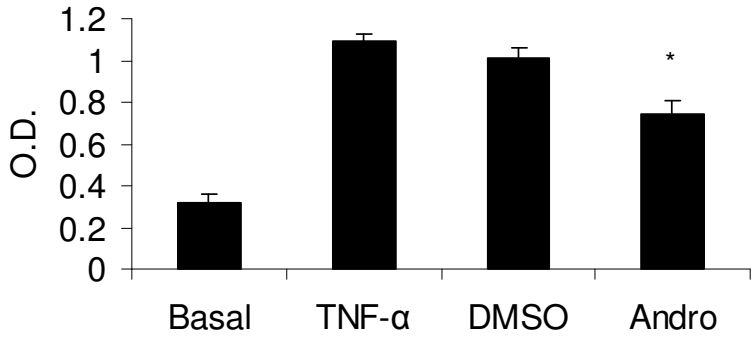


Figure 5.17 Effects of andrographolide on TNF-α-induced p65 DNA-binding activity in A549 cells. A549 cells were incubated with or without andrographolide (30 μM) for 4 hours and then stimulated with TNF-α (10 ng/ml) for 30 min. Nuclear proteins of cells were isolated and analyzed by TransAM assay. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $P < 0.05$.

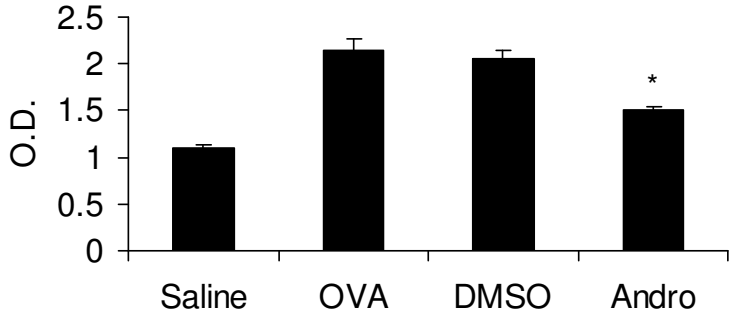


Figure 5.18 Effects of andrographolide on p65 DNA-binding activity in lung tissue. Lung tissues were collected 24 hours after the last OVA or saline aerosol challenge pretreated with either DMSO or andrographolide (1mg/kg). Nuclear protein was isolated and DNA-binding activity was measured by TransAM assay. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $P < 0.05$.

Disruption of p65 or IKKβ genes causes embryonic lethality in mice due to severe liver degeneration (Beg et al., 1995; Li et al., 1999b). To further investigate the toxicity of andrographolide on the function of liver due to the inhibition of IKKβ activity, we measured the activities of serum ALT and AST, indicators of hepatocellular injury, using a kinetic spectrophotometric assay. Andrographolide did affect the activities of both ALT and AST (Figure 5.19).

5.1.10. Effects of andrographolide on activities of MAP kinases in vitro

Previously, several studies indicated andrographolide might exert its anti-inflammatory effects by inhibiting the ERK MAP kinase pathway (Liang et al., 2008; Qin et al., 2006). To investigate the possible mechanisms of andrographolide in ERK pathway, we also tested if andrographolide affects TNF-α-induced the phosphorylation of MEK1/2 and ERK1/2 in the epithelial cells with the pretreatment of DMSO or andrographolide. Interestingly, andrographolide markedly inhibited TNF-α-induced the phosphorylation of MEK1/2 and slightly reduced the phosphorylation of ERK1/2 in epithelial cells compared with control (Figure 5.20). These results suggest andrographolide also can affect the MAP kinas pathway, although whether the inhibition of MEK and ERK by andrographolide is related to the inhibition of the phosphorylation of IKKβ still remains unclear.

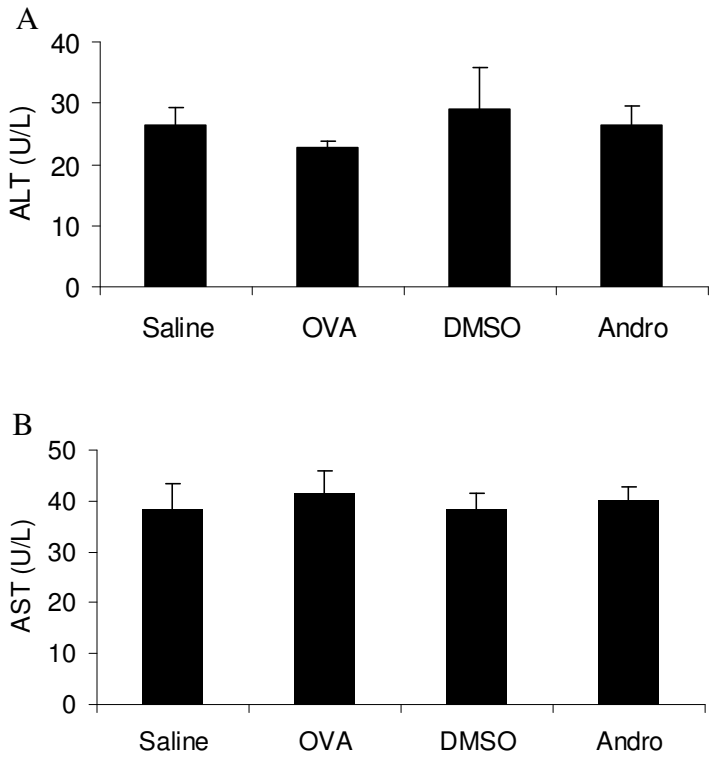


Figure 5.19 Effects of andrographolide on the activities of serum ALT and AST. Mouse serum was collected 24 hours after the last OVA or saline aerosol challenge pretreated with either DMSO or andrographolide (1 mg/kg). The activities of (A) ALT and (B) AST were analyzed using a kinetic spectrophotometric assay (n = 5 – 6 mice per group). Values shown are the mean ± SEM. *Significant difference from DMSO control, $P < 0.05$.

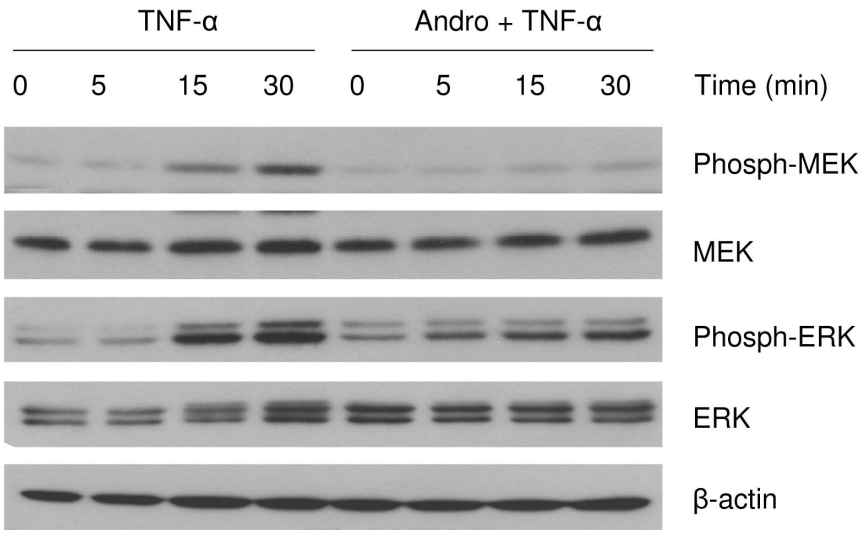


Figure 5.20 Effects of andrographolide on TNF-α-induced MEK and ERK activation in A549 cells. A549 cells were incubated with or without andrographolide (30 μM) for 4 hours and then stimulated with TNF-α (10 ng/ml) for the indicated times. Total proteins of cells were isolated and analyzed by Western blotting with the indicated antibodies. β-actin was used as an internal control. The experiments were repeated for three times with similar pattern of results.

5.2. Discusssion

The medicinal plant *andrographis paniculata* has been widely used for a variety of diseases in Asia for a long time (Negi et al., 2007). The diterpene compound andrographolide is the major component in this plant and has been shown to possess anti-inflammatory activities (Panossian et al., 2000; Wang et al., 2004; Wang et al., 2007). Andrographolide has been shown to suppress complement-5a induced chemotactic cell migration (Tsai et al., 2004), reduce the production of TNF-α and IL-12 in LPS-stimulated murine peritoneal macrophages (Qin et al., 2006), and inhibit partially the IL-2 production induced by Con-A in murine T cells (Burgos et al., 2005), probably via the inhibition of extracellular signal-regulated kinase1/2 (ERK)1/2. Andrographolide may also prevent reactive oxygen species in human neutrophils via interference of the protein kinase C-dependent pathway (Shen et al., 2002). Moreover, andrographolide is able to interfere with T cell proliferation and cytokine release in response to allogenic stimulation, through the blockade of dendritic cell maturation and their ability to present antigens, suggesting an immuno-modulatory activity of andrographolide (Iruretagoyena et al., 2005). Although there are numerous reports on the pharmacological activities of andrographolide, the detailed molecular mechanisms remain unclear. Recently, andrographolide has been found to attenuate inflammation by inhibiting the NF-κB pathway via covalent modification of reduced cys^{62} of the p50, one subunit of NF-κB (Wang et al., 2007; Xia et al.,

2004). Because a variety of NF-κB-dependent genes, such as iNOS, VCAM-1, ICAM-1, TNF-α, and IL-6, have found to be modulated by andrographolide, it is highly likely that andrographolide may exert its anti-inflammatory effects via modulating NF-κB pathway.

NF-κB activity is up-regulated in allergic airway inflammation both in human and in animal models of asthma (Broide et al., 2005; Donovan et al., 1999; Gagliardo et al., 2003; Hart et al., 1998; Poynter et al., 2004). Various therapeutic strategies targeted at the NF-κB signaling pathway such as NF-κB specific decoy oligonucleotide (Desmet et al., 2004), p65-specific antisense oligonucleotide (Choi et al., 2004), and IKK-2 selective small molecule inhibitors (Birrell et al., 2005) have demonstrated beneficial effects in experimental asthma models. Therefore, we investigated the potential anti-inflammatory effects of andrographolide in an asthma mouse model and its molecular mechanisms of action.

Asthma is a Th2-polarized chronic inflammatory disease (Meyer et al., 2008). Asthmatic patients are genetically predisposed toward the differentiation of naïve T cells into Th2 cells and away from the Th1 cells (Mazzarella et al., 2000). CD4+ Th2 cells play an essential role in the pathophysiology of the allergic airway inflammation (Herrick and Bottomly, 2003). Th2 cytokines, including IL-4, IL-5, and IL-13, can be produced by various resident cells such as bronchial epithelial cells, mast cells, and alveolar macrophages, as well as by infiltrated

inflammatory cells such as lymphocytes and esosinophils. IFNγ is the main Th1 cytokine and its production is inhibited by Th2 cytokines such as IL-4 and IL-13 (Mazzarella et al., 2000). NF-κB is a critical transcription factor for Th2 cell differentiation, via regulation of GATA-3, which is uniquely expressed in Th2 cells (Das et al., 2001). Moreover, it has been shown that NF-κB is necessary for the induction of IL-13 upon the cross-linking FcεRI in human mast cells (Lorentz et al., 2003). Our data show that andrographolide dramatically reduced the levels of IL-4, IL-5, and IL-13, but slightly increased IFNγ levels in BAL fluid from OVA-challenged mice, in a dose-dependent manner. These findings are consistent with the opposing profile of Th1/Th2 paradigm in asthma. Several studies, on the inhibition of the NF-κB function in OVA-challenged mice using conditional knockout of IKKβ transgenic IκBα mutant expression selectively in airway epithelium (Broide et al., 2005; Poynter et al., 2004), or aerosol administration of NF-κB decoy oligonucleotide (Desmet et al., 2004), showed a similar inhibitory pattern of cytokine profile as our presenting findings, suggesting that andrographolide may exert its anti-inflammatory effects via inhibition of the NF-κB signaling pathway. Antigen receptor activation in T and B cells and in mast cells has been shown to culminate in NF-κB activation (Li et al., 2004; Peng et al., 2005; Weil and Israel, 2004), and andrographolide may modulate NF-κB mediated cytokine production in these inflammatory cells. In addition, airway smooth muscle is another source of proinflammatory cytokines and may also promote

recruitment, activation, and migration of inflammatory cells during the airway inflammation (Howarth et al., 2004; Tliba et al., 2008). Repression of NF-κB signaling pathway has been shown to block IL-13-induced eotaxin production in cultured human airway smooth muscle cells (Birrell et al., 2005). As such, the observed reduction of IL-4, IL-5, and IL-13 levels in BAL fluid from andrograhpolide treated mice may be due to inhibition of NF-κB activation in those inflammatory and airway resident cells.

The recruitment of inflammatory cells, particularly eosinophils, into the airways is one of the hallmarks of asthma (Kelly et al., 2007; Rothenberg and Hogan, 2006). Our findings showed that andrographolide prevented the infiltration of inflammatory cells, such as eosinophils and lymphocytes, into the airways, in a dose dependent manner, as shown by a significant drop in total cell counts and differential cell counts in BAL fluid. In agreement with BAL fluid cell count data, there was also a reduction in the infiltration of inflammatory cells into peribronchiolar and perivascular tissues shown by histological examinations. The recruitment of inflammatory cells from circulation into the airways is a dynamic and multistep process which includes rolling, adhesion, and transmigration (Kelly et al., 2007; Ley et al., 2007; Medoff et al., 2008). Rolling is mediated by selectins, which interact with glycoprotein ligands. Chemokines are believed to play a key role in triggering adhesion of inflammatory cells. They activate integrins expressed on inflammatory cells, which bind to adhesion molecules, such as

ICAM-1 and VCAM-1, expressed on endothelial cells, leading to the adhesion of inflammatory cells. The mechanisms of migration still remain unclear. A wide variety of proteins, including CD99 and junctional adhesion molecules, have been involved in this migration process. In asthma, trafficking of inflammatory cells into airways is orchestrated by Th2 cytokines, including IL-4, IL-5, and IL-13, eotaxins, and adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin (Jia et al., 1999; Kelly et al., 2007; Ono et al., 2003; Rothenberg and Hogan, 2006). IL-4 and IL-13 can induce the expression of VCAM-1 in endothelial cells, facilitating the recruitment of inflammatory cells in asthma (Bochner et al., 1995). IL-13 is also a potent stimulator of chemokines, including eotaxin-1, eotaxin-2, and MCP-1, in the airways (Li et al., 1999a; Zhu et al., 2002). We demonstrated that andrographolide slightly down-regulated the expression of VCAM-1, ICAM-1, remarkedly decreased the level of E-selectin in OVA-challenged lungs, and significantly reduced eotaxin-1 levels in BAL fluid. These results are likely to be due to an induced inhibition of NF-κB pathway by andrographolide, as the gene expression of VCAM-1, ICAM-1, E-selectin, and eotaxins, are all regulated by the NF-κB pathway (Kumar et al., 2004). Taken together, the observed reduction of inflammatory infiltration in airways may be a composite effect of the reduction of IL-13 and the decrease in the expression of eotaxin and adhesion molecules, secondary to the blocking of NF-κB pathway by the treatment of andrographolide.

Mucus hypersecretion, including excessive mucus secretion, goblet cell hyperplasia, and submucosal gland hypertrophy, is a consistent feature in asthma (Morcillo and Cortijo, 2006). Mucus hypersecretion also contributes to airflow limitation and AHR, and are associated with high morbidity and mortality in uncontrollable asthma patients (Morcillo and Cortijo, 2006). We found that andrographolide significantly reduced goblet cell hyperplasia and excessive mucus secretion shown by histological examinations, as compared with DMSO control. There is cumulative evidence to support the pivotal role of IL-13 in the mucus hypersecretion observed in asthma (Wills-Karp, 2004). Blockade of IL-13 alone has been shown to prevent the mucus hypersecretion in asthma mouse model (Wills-Karp et al., 1998; Zhu et al., 1999). Components of IL-13 signaling pathway such as STAT-6, also have been shown to be involved in the production of mucus (Gavett et al., 1997; Kuperman et al., 1998). The role of IL-5 in the mucus hypersecretion remains unclear. It has also been proposed that the expression of IL-5 in the airway alone is sufficient to induce CD4(+) T cell-dependent goblet cell metaplasia in a study of a pulmonary IL-5 transgenic mouse model (Justice et al., 2002). However, IL-4- and IL-13-deficient but not IL-5-deficient mice were protected from airway mucin changes after chronic allergen exposure (Leigh et al., 2004). NF-κB pathway also plays an important role in the mucus hypersecretion and selective ablation of NF-κB function in airway epithelium inhibited OVA-induced mucus production in mice (Broide et al., 2005;

Desmet et al., 2004; Poynter et al., 2004). Interestingly, the Muc5ac gene, which is implicated in the production of gel-forming mucins in the airway, is regulated by a IKKβ dependent mechanism in epithelial cells following the stimulation with TNF-α (Lora et al., 2005). We also observed a drastic drop in the level of Muc5ac mRNA by the treatment of andrographolide in OVA-challenged asthma mouse. Therefore, the dramatic decrease in mucus production of airway in the andrographolide-treated mice may be attributed to a substantial drop in Th2 cytokines, particularly IL-13. Moreover, the inhibition of NF-κB pathway by andrographolide may affect mucus production via an indirect way through regulation of the Muc5ac gene.

Elevated serum IgE and IgG1 are the hallmarks of a Th2 immune response in mice (Avila, 2007; Okahata et al., 1990; Vance et al., 2004). Our data showed that serum levels of total IgE, OVA-IgE and OVA-IgG1 were greatly decreased by andrographolide, whereas no significant inhibition was observed in OVA-IgG2a, an indicator of Th1 response. NF-κB pathway has been found to be involved in the differentiation and maturation of B lymphocytes (Matthias and Rolink, 2005). Selective p50- or p52- deficient mice have exhibit malfunction of B lymphocytes (Cariappa et al., 2000; Franzoso et al., 1997). B lymphocytes produce OVA-specific IgE or IgG after undergoing Ig class switching. This process in asthma requires two signals, one of which is specific antigen and cytokines such as IL-4 and IL-13, by IL-4 receptor α chain via STAT-6 pathway (Emson et al., 1998;

Wills-Karp, 2004). The second signal is the interaction between CD40 on B cells and CD40L on activated Th cells. The biological activities of IgE are mediated by its interaction with FcεRI on mast cells and basophils (Kawakami and Galli, 2002). Crossing linking of IgE and FcεRI lead to degranulation of mast cells and release of histamines, leukotrienes, and prostaglandins, initiating the multiple signaling cascades in asthma . These mediators also act on smooth muscle cells, causing acute bronchoconstriction which initiate the early-phase reactions of asthma. It has been shown that NF-κB may also be involved in the IgE-mediated mast cell activation (Lorentz et al., 2003; Peng et al., 2005). Therefore, the observed reduction in serum total IgE, OVA-IgE, and OVA-IgG1 in our asthma model by the treatment of andrographolide may be due to its inhibitory effects on B cell activation, Th2 cytokine production from inflammatory cells via NF-κB pathway.

Chitin is a polymer of N-acetylglucosamine and is the one of the components of the cell walls of fungi and exoskeletal elements worms and arthropods, but except mammals (Elias et al., 2005). Chitinases are a group of enzymes that break down chitin structures in organisms. Previously, chitinases were only considered as part of innate immune response of host againt a chitin-containing pathogen. Recently, a family of chitinase proteins including AMCase, Ym1, Ym2, YKL-40 has been found to be dramatically increased in allergic airway inflammation and to be involved in the pathophysiology of asthma (Chupp

et al., 2007; Elias et al., 2005; Moffatt and Cookson, 2008; Webb et al., 2001; Zhao et al., 2005; Zhu et al., 2004). One study has shown that AMCase is induced in a IL-13-dependent manner in epithelium and macrophages in an asthma mouse model and is increased in asthma patients as compared with normal controls (Zhu et al., 2004). When given intratracheally, IL-13 increased Ym1 and Ym2 levels in BAL fluid from mice (Webb et al., 2001). YKL-40, also called chitinase-3-like-1, is elevated in the serum of asthma patients and is correlated with severity of asthma (Chupp et al., 2007). Moreover, the gene that encodes YKL-40 is considered a susceptibility gene for asthma by a large population study (Ober et al., 2008). Although the mechanisms of chitinases in asthma remain unclear, they may trigger immune responses upon antigen challenge, act directly as a kind of chemotactic agents, regulate tissue inflammation and remodeling, or work just as a biomarker for asthma (Elias et al., 2005). Our data have showed that andrographolide moderately inhibited the expression of AMCase and Ym-1, but greatly suppressed Ym-2 and YKL-40 in the OVA-challenged lungs as compared with vehicle control. The reduction of chitinases, especially Ym-2 and YKL-40, by the treatment of andrographolide may be a result of decreased level of IL-13 in the airways. Furthermore, AMCase contributes to the Th2-polarized inflammation (Zhu et al., 2004). It also has been shown that Ym-1 protein is a potent chemotactic agent for eosinophils and CD4+ T cells (Owhashi et al., 2000). The

decreased chitinases may further lead to diminished infiltration of inflammatory cells in the lung.

NO is a ubiquitous gaseous molecule that regulates airway and vascular smooth muscle tone. There has been shown that the increased level of NO is associated with the severity of asthma and may reflect allergic inflammation in the airways (Kharitonov et al., 1995; Massaro et al., 1995). Furthermore, inhaled corticosteroids can dramatically reduce the level of exhaled NO in asthma patients (Kharitonov et al., 1996a; Kharitonov et al., 1996b; van Rensen et al., 1999). NO is synthesized by three different oxide synthases, of which the most important one in the airway is iNOS. iNOS is induced by proinflammatory cytokines in a variety of cells including macrophages and epithelium. The iNOS was found to be elevated in the airway epithelium and macrophages in asthma patients (Hamid et al., 1993). The increased level of iNOS may be due to the effects of proinflammatory cytokines, oxidants, and other inflammatory mediators. Although some studies have supported the role of NO and iNOS in asthma, the mechanisms of NO and iNOS remain controversial and unclear (Di Maria et al., 2000; Fischer et al., 2002; Ricciardolo, 2003). Cumulative evidence has proved that the gene of iNOS is regulated by the NF-κB pathway (Pahl, 1999). In addition, IL-13 was found to induce iNOS derived NO in normal human bronchial epithelial cells (Chibana et al., 2008; Suresh et al., 2007). Consistent with previous data, level of iNOS in lung tissue of our asthma model was elevated. The reduced level of iNOS

by the treatment of andrographolide may be due to the inhibition of NF-κB pathway and reduced IL-13 level in airways.

TSLP is an epithelial cell-derived cytokine and play a key role in the initiation and maintenance of asthma in a dendritic cell- and mast cell-mediated Th2 immune response (Allakhverdi et al., 2007; Liu, 2007; Rochman et al., 2007). The expression of TSLP is increased in the asthmatic airways and correlates with the severity of asthma (Ying et al., 2005). Furthermore, it has been shown that TSLP is regulated by a NF-κB-dependent manner in human airway epithelia cells and the promoter of the mouse TSLP gene contains an orthologous NF-κB binding site (Lee and Ziegler, 2007). Our data have shown that andrographolide remarkedly inhibited the expression of TSLP in the lung tissue of asthmatic mouse as compared with vehicle control. This reduction of TSLP level may be due to the inhibition of NF-κB pathway by andrographolide and further contribute to the decreased Th2 cytokines production in airways.

It is believed that inflammatory mediators released during the allergic inflammation play a critical role in AHR development (Cohn et al., 2004; Fernandes et al., 2003). We report here that andrographolide significantly inhibited OVA-induced AHR to increasing concentrations of methacholine. IL-5 is responsible for the differentiation, maturation, and activation of eosinophils, and plays a critical role in AHR by mobilizing and activating esoinophils, leading to the release of pro-inflammatory products such as major basic protein and

cysteinyl-leukotrienes, which are closely associated with AHR (Rothenberg and Hogan, 2006). Blockade of IL-4 receptor has been shown to inhibit the allergen-induced AHR in mice and inhaled IL-4 induced AHR in response to methacholine in a human placebo-controlled study, indicating that IL-4 can increase AHR in asthma (Gavett et al., 1997; Shi et al., 1998). In addition, IL-13 has been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated in AHR (Vargaftig and Singer, 2003; Wills-Karp, 2004). Moreover, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (Lorentz et al., 2003). Thus, the observed reduction of AHR may be associated with reduction in Th2 cytokine production, tissue eosinophilia, and serum IgE level by andrographolide.

We found that treatment with andrographolide inhibited OVA-induced p65 DNA-binding activity in the lung tissue, which is consistent with anti-inflammatory effects of andrographolide in the asthma model. To further study how andrographolide interferes with NF-κB pathway, we investigated the effects of andrographolide on TNF-α-induced NF-κB activation in A549 human epithelial cells. We found that andrographolide inhibited TNF-α induced phosphorylation of IKKβ, leading to the decreased phosphorylation of IκBα, delayed degradation of IκBα, reduced translocation of p65, diminished DNA-binding activity of p65. Activation of IKK complex depends on ubiquitination of NEMO, phosphorylation

of IKKβ and IKKα, and IKK multimerization in response to stimuli such as TNF-α, in the canonical pathway (Hacker and Karin, 2006). The activated IKK complex then phosphorylates IκBα, causing the ubiquitination and following degradation of IκBα. Following the degradation of IκBα, NF-κB subunit p65 translocates into the nucleus and activate the expression of targeted genes (Perkins, 2007). Therefore, andrographolide may exert its anti-inflammatory effects via inhibition of the phosphorylation of IKKβ. IKK complex subunits also have various substrates. Activation of IKKβ can lead to the phosphorylation of p65 at Ser^{536} and its nuclear translocation, enhancing p65 transactivation functions by promoting interactions with coactivator proteins, such as p300 and CBP (Perkins, 2007; Sizemore et al., 2002). We postulate that the reduction in the phosphorylation of p65 at Ser^{536} after the treatment of andrographolide in epithelial cells may be due to the inhibition of the phosphorylation of IKKβ.

Because previously there are many reports about the effects of andrographolide on the ERK MAP kinase pathway, we also tested TNF-α-induced phosphorylation of MEK1/2 and ERK1/2 in the epithelial cells with the pre-treatment of DMSO or andrographolide. Interestingly, we found the reduced phosphorylation of MEK1/2 and its downstream ERK1/2 after the treatment of andrographolide in epithelial cells. It has been shown that there are also links between IKKβ and the MAP kinase pathway (Perkins, 2007). In resting cells, the MEK kinase (tumor progression locus 2 [TPL2]), which is required for the TNF-α

induced MEK and ERK activation (Eliopoulos et al., 2003), forms a complex with one NF-κB subunit p105, which is the precursor of p50 (Belich et al., 1999; Waterfield et al., 2003). The activation of IKKβ may induce the proteolysis of p105 or phosphorylaiton of TPL2 at Thr^{290}, which in turn leads to the release of bound TPL2 from the complex, causing the activation of MEK and ERK pathway (Beinke et al., 2004; Cho et al., 2005; Cho and Tsichlis, 2005; Waterfield et al., 2004). Therefore, the reduced phosphorylation of MEK1/2 and ERK1/2 may be due to the inhibited IKKβ activity or direct inhibition of phosphorylation of MEK1/2 by the andrographolide.

Allergic airway inflammation is a complex disorder, involving a wide array of inflammatory cells and mediators. We report that andrographolide effectively inhibited OVA-induced Th2 cytokines and eotaxin production, pulmonary infiltration of inflammatory cells, serum IgE and IgG1 synthesis, mucus hypersecretion, and AHR in a mouse asthma model, via the inhibition of NF-κB pathway. These finding support that andrographolide, as an active ingredient of herb medicinal, may have potential in the treatment of asthma.

Our data have shown the efficacy of andrographolide in the asthma mouse model. However, there are still some questions regarding the potential use of andrographolide in asthma patients. First, the toxicity of andrographolide should be tested in asthma patients. Although andrographis paniculata has been used as a herbal medicine for a long time in Asia and Scandinavia, the pharmacokinetics of

andrographolide in asthma patients remains unknown. Second, the effects of andrographolide, combined with inhaled corticosteroids or long-acting β2 agonist, in asthma mouse model remain unclear. Third, we will precede the andrographolide to clinical trials for asthma patients, if the toxicity of drug and proper dose of drug in human patients are confirmed by preliminary experiments. The efficacy of andrographolide in different genotype or phenotype of the particular asthma subtype might be assessed in the future. In brief, although andrographolide is a promising herbal drug for asthma patients, there are still more questions than answers about the toxicity, efficacy, and mechanisms of andrographolide.

6. CONCLUSION

The NF-κB family is essential in coordinating both innate and adaptive immunity and is involved in the regulation of a broad array of genes in response to diverse stimuli. This study demonstrates the potential anti-inflammatory effects of a GSK-3β inhibitor, TDZD-8, and a herbal medicinal, andrographolide in a mouse asthma model and elucidates their mechanisms of action in the regulation of NF-κB pathway.

This study demonstrates that TDZD-8 is effective in blocking phosphorylation of p65 at Ser^{536} in an asthma model and in TNF-α-stimulated normal human bronchial epithelial cells. TDZD-8 inhibited ovalbumin-induced total cell counts, eosinophil counts, Th2 cytokines and eotaxin production, serum OVA-IgE, pulmonary infiltration of inflammatory cells, mucus hypersecretion in lungs, AHR to methacholine in an asthma mouse model, probably via the inhibition of the phosphorylation of NF-κB subunit p65.

Accordingly, we examined anti-inflammatory effects of another potential NF-κB inhibitor, in an asthma mouse model. Andrographolide, given intraperitoneally, markedly inhibited OVA-induced airway and lung tissue infiltration of inflammatory cells, Th2 cytokines and chemokines release, serum total and specific IgE and IgG1, airway mucus production, expression of the inflammatory molecules, and AHR in the asthma model. Further experiments for the effects of andrographolide in epithelial cells revealed that andrographolide reduced the TNF-α induced phosphorylation of IKKβ, causing the decreased p65

DNA-binding activity and diminished phosphorylation of p65. These results suggest that specific regulation of the phosphorylation of key molecules in the NF-κB pathway may provide a significant advance in the treatment of asthma.

This study indicates the regulation of posttranscriptional activity in the NF-κB pathway such as phosphorylation of IKKβ and p65, by TDZD-8 and andrographolide exhibits anti-inflammatory effects in asthma model as compared with direct inhibition of IKKβ kinase activity or expression of p65. Taken together, the present findings implicate that appropriate and specific inhibition of signaling molecules that regulate the NF-κB pathway may have therapeutic potential for the treatment of allergic airway inflammation, without compromising immunity and cell survival, thereby avoiding potential side effects.

In the future, we may need to do more research about the mechanisms and clinical efficacies of these two drugs. Because phosphorylation of the components of NF-κB pathway is a dynamic process, more studies need to be done to investigate the precise regulation mediated by these two drugs. Combination of any one of these drugs with inhaled steroids may reveal their synergistic effects in asthma. Understanding multiple mechanisms of these two drugs and their effects in steroid-resistant asthma might show their unique roles in refractory asthma besides anti-inflammatory effects. Finally, clinical trials are needed to confirm the safety and efficacy of TDZD-8 and andrographolide in asthma patients.

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Appendix

List of Reagents and Solutions

Sensitization solution for mouse asthmatic model

OVA (grade Ⅴ)	20 μg
$Al(OH)_3$	4 mg
Saline	0.1 ml

Challenge solution (15 ml)

OVA (grade Ⅴ)	0.15 g
Saline	15 ml

Modified Wright's staining (Liu's staining)

Liu A	Eosin Y	0.18 g
	Methylene blue	0.05 g
	Methanol	100 ml
Liu B	Methylene blue	0.7 g
	Azur Ⅰ	0.6g
	$Na_2HPO_4 \cdot 12H_2O$	12.6 g
	KH_2PO_4	6.25 g
	H_2O	500ml

Red cell lysis buffer for BAL fluid cell counts (2 ml)

NH_4Cl	0.0175 g
H_2O	2 ml

Diluent buffer for BAL fluid cell counts (4 ml)

RPMI	4 ml
BSA	0.04 g

Sample lysis buffer (Western Blotting)

Tris-HCl pH7.4	75 mM
NaCl	150 mM
Triton X-100	1%
Glycerol	10%
Protease inhibitor cocktail	1X
Dilution	
NaF	20 mM

10% APS (fresh preparing)

APS	20 mg

H2O	200 µl

5X Sample Buffer

3 M Tris-HCl pH 6.8	312.5 mM
Glycerol	50%
2-mercaptoethanol	25%
SDS	10%
1% Bromophenol blue	0.0625%

10% SDS (100 ml)

Sodium dodecyl suphate	10 g
H_2O	100 ml

Running gel (15 ml for 2 gels)

1.5 M Tris-HCl pH8.8	3.73 ml
H_2O	5.96 ml
30% Acrylamide (37.5 : 1)	4.93 ml
10% SDS	0.15 ml
10% Glycerol	0.15 ml
10% APS	0.077 ml
TEMED	0.0073 ml

Stacking gel (5 ml for 2 gels)

0.5 M Tris-HCl pH6.8	1.25 ml
H_2O	3 ml
30% Acrylamide (37.5 : 1)	0.625 ml
10% SDS	0.5 ml
10% Glycerol	0.5 ml
10% APS	0.015 ml
TEMED	0.005 ml

10 X Electrophoresis buffer (2 liter)

Tris-Base	60.57 g
glycine	288.27 g
SDS	20 g
H_2O (top up)	2000 ml

5X Transfer buffer for PVDF (1 liter)

Tris-Base	60.57 g
Glycine	72.07 g
H_2O (top up)	1000 ml

PBS solution (10 liter)

NaCl	80 g
Na_2HPO_4	11.6 g
KH_2PO_4	2 g
KCl	2 g
Adjust pH to 7.2 – 7.4	
H_2O (top up)	10 liter

10X TBS (Tris-buffered saline) (1 liter)

Tris-HCl pH7.5	121.4 g
NaCl	90 g
H_2O (top up)	1000 ml

Tris buffered saline-Tween 20 (TTBS) (2 liter)

TBS	2000 ml
Tween 20	1 ml

Blocking buffer fore western blotting (100 ml)

Non-fat milk powder or BSA	5 g
TTBS	100 ml

AP substrate solution (15 ml)

25 × AP buffer	600 µl
BCIP	150 µl
NBT	150 µl
H_2O	14.1 ml

HRP substrate solution (1ml)

ECL reagent 1	0.5 ml
ECL reagent 2	0.5 ml

Stopping solution (ELISA)

1 M H_2SO_4

Reverse transcription cocktail (30 µl)

AMV 5X buffer	6 µl
dNTP buffer (10 mM)	1.5 µl
Rnasin ribonclease inhibitor (40 U/µl)	0.75 µl
AMV-reverse transcriptase (10 U/µl)	2 µl
Random primer (3 µg/µl)	0.33 µl
RNA	1 µl

DEPC H_2O (top up)	30 µl

PCR reaction cocktail (25 µl)

PCR master mix 2X	12.5 µl
Upstream primer (10 µM)	1 µl
Downstream primer (10 µM)	1 µl
Nuclease Free H_2O	9.5 µl
cDNA template	1 µl

Agarose gel solution (100 ml)

Agarose powder	2 g
1X TAE (0.04 M Tris-acetate and 0.001 M EDTA)	100 ml

10X DNA loading buffer (20 ml)

Bromophenol blue	0.025 g
Xylene cyanol	0.025 g
Glycerol	12.5 ml
H_2O	7.5 ml